AD 407 298

63-4-1

S-608 - Rpt #7(Final)
Contract: DA19-129-qm-1740
Southwest Research Inst.

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Study of Free Radicals in Irradiated Beef

Period: 30 December 1960 - 29 December 1962

ARMED FORCES FOOD AND CONTAINER INSTITUTE
U. S. Army Quartermaster Research and Engineering Center
Chicago 9, Illinois

CONTRACT RESEARCH PROJECT REPORT

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Project No.: 7-84-01-002
Contract: DA19-129-QM-1740 (OI 5054)
File No.: S-608
Report No.: 7 (Final)
Period: 30 Dec 1960
29 Dec 1962
Initiation Date: 30 Dec 1960

Title of Contract: STUDY OF FREE RADICALS IN IRRADIATED BEEF

SUMMARY

~~An investigation having as its objective~~ a description of the mechanism by which wet dog hair odor is induced in cooked beef by radiation is described. The investigation was largely of the non-dialyzable fraction of aqueous extract of cooked beef, prepared according to Paul A. Hedin and his group at QMF&CI. Electron spin resonance was the principal method employed. Although the results do not afford a substantial basis for conclusions, the electron spin resonance data from this program, together with data from other investigators, suggest that glycine and methionine units in the protein, either bonded to one another or as close neighbors, make up a major source of wet dog hair

odorants through a reaction involving a radiation-induced free radical and water. There are a number of proteins and peptides with known amino acid sequences; irradiation and odor testing of certain of these that incorporate a variety of relative locations of glycine and methionine units should serve to verify or refute this suggestion. No methanol extractable odor precursor seems to exist in Hedin's material, but the wet dog hair odorants themselves do seem extractable following irradiation of Hedin's material.

I. INTRODUCTION

The objective of the program has been a description of the mechanism of the production of wet dog hair (WDH) odor induced by the gamma irradiation of beef. Toward this end a model system has been investigated that had previously been studied extensively by Paul A. Hedin and his collaborators at the Quartermaster Food and Container Institute, the nondialyzable fraction of hot aqueous extracts from beef chuck (Refs. 1, 2, 3). The principal method of investigation has been electron spin resonance (ESR), since a mechanism of odor production involving radiation-induced free radicals has been considered likely by Hedin.

In the remainder of this Introduction, a background for an understanding of the problem will be drawn from literature that has reached this laboratory, principally from Hedin's laboratory at QMF&CI; from W. A. Landmann's group at the American Meat Institute Foundation; and from a group at the Massachusetts Institute of Technology, headed by B. E. Proctor, and more lately by S. A. Goldblith. This will be followed by a restatement of the problem.

The group at MIT has been doing concurrent radiation and distillation of odorants from beef slurries, milk, and certain food

constituents, with subsequent identification of the trapped odorants (Refs. 4, 5). A number of volatile substances trapped from enzyme inactivated irradiated beef have been identified positively. Some are alcohols--ethanol, n-butanol, 2-butanol, and n-pentanol--which are also found in unirradiated beef. From both irradiated and unirradiated beef come acetoin and n-hexanol, but from irradiated beef only arise the aldehydes n-heptanal, n-octanal, n-nonanal, and methional. Additionally, ethyl acetate, 2-butanone, n-nonane, 1-nonene, n-undecane, and 1-undecene arise from the irradiated beef only. The volatiles that have been identified were collected in an ice bath and probably also in a dry ice-ethanol bath, for in these baths were trapped nearly all of the distillate. Additional vapors were trapped in liquid nitrogen, but the odorants in the liquid nitrogen traps have not yet been identified. Recombination of fractions obtained by temperature programmed vapor phase chromatography of the combined trapped substances gave a "rather good representation" of the WDH odor.

While the MIT group is investigating the identity of the odorants (the reaction products), the group at the American Meat Institute Foundation is investigating the identity of the materials in meat which, when irradiated, give rise to the WDH odor (the reactants or precursors) (Ref. 6). More specifically, the group is attempting to identify a single precursor, a pure substance, which is responsible for the WDH odor. Irradiated meat has been extracted with water and the residue

has been lyophilized. The dry residue has been extracted with hexane and the residue from this extraction has been air dried and re-extracted with chloroform-methanol. The chloroform-methanol extract has been dried at room temperature with a water jet aspirator and this crude extract has been chromatographed on a silicic acid-chloroform column using continuous gradient methanol elution. One fraction, Fraction 5, gave the WDH odor, while the fraction immediately following it gave only a trace of the odor. Fraction 5 was characterized as the phospholipid fraction, part of which was ninhydrin positive and gave a blue fluorescence. A part, however, was ninhydrin negative and gave a yellow fluorescence. A similarly obtained fraction from unirradiated beef seemed to contain only ninhydrin positive material, giving a blue fluorescence. A chloroform-methanol solution of Fraction 5 could, on standing, yield a white precipitate which developed a strong WDH odor in aqueous solution. The dried white precipitate was found to contain nitrogen, but neither sulfur nor phosphorus. It was ninhydrin negative before and after acid hydrolysis and as much as 5.6% sugar seemed present. An ultraviolet absorption spectrum of the aqueous solution showed a maximum at 258 mμ, with a minimum at 230 mμ, which was considered strongly suggestive of a nucleic acid; but the presence of a nucleic acid could not be determined with certainty.

A fractionation of unirradiated beef was carried out in the same manner, and the fractions were irradiated in aqueous suspension.

Fraction 5 developed WDH odor as it had from irradiated beef, but Fraction 7, which had been allowed to stand overnight before diluting with water, gave a more intense WDH odor. A portion of Fraction 5 left unirradiated in the dry state was found to develop the ability to give WDH odor upon adding water after standing for three weeks at room temperature.

Certain of Hedin's work on the ammonium sulfate fractionation of the nondialyzable fraction of hot aqueous extract from cooked beef was repeated for comparison, but the group was unable to complete enough work to decide whether the fraction of Hedin's material giving the more intense WDH odor contained the material in the phospholipid fraction that had been isolated. Hedin's group at QMF&CI had found that the nondialyzable fraction of hot aqueous extracts from cooked beef could be fractionated further by ammonium sulfate precipitation to obtain a fraction giving a relatively intense WDH odor compared with fractions obtained at other saturation levels. This was the 26 - 50% fraction, largely protein, which was at least 90% electrophoretically homogeneous. Irradiation of the dry material was done following the fractionation, subsequent suspension in water giving the WDH odor.

The unfractionated nondialyzable preparation had been found nearly totally protein, with 16.04% nitrogen, and 0.76% ash. Of the ash, 0.21% was phosphorus, and 0.021% was iron. On the basis of this, 5% had been considered nucleoprotein. Irradiation of the unfractionated material reduced the apparent cysteine and methionine content to zero

and decreased the histidine content by 25%. Irradiating a lyophilized sample to a dose of 5 megarads produced little odor while the material was left dry; but when water was added, the typical WDH odor developed. Irradiating 33% slurries to doses ranging from 1 to 10 megarad at 1 megarad intervals and performing an odor analysis through an informal panel led to the conclusion that above a 3 or 4 megarad dose the quantity of odor did not increase. Further, a series of samples that had received 1 - 10 megarad were dialyzed against a 10% sodium chloride solution to remove the radiation odor, were then lyophilized, and were re-irradiated in the dry state to a dose of 5 megarad before re-examination. Those samples that had received more than 2 megarad on the first irradiation developed little or no odor upon the addition of water. The odor was found to be quenched by sodium p-chloromercuribenzoate, n-ethylmaleimide, and mercuric acetate.

With respect to the ammonium sulfate fractions, the loss of nearly all the sulfhydryl groups in the 26 - 50% fraction with a 5 megarad dose was again demonstrated, but the loss of sulfur was negligible. Irradiation was found to increase the absorption of the 26 - 50% fraction at 260 mμ and the absorptive centers were found partially dialyzable. However, this increase in absorption was demonstrated by the other fractions which did not give the intense WDH odor as well, leading to the conclusion that the effect was unrelated to odor production. Amino acid analysis showed that the cystine had been destroyed, that methionine sulfoxide

had been produced, and that some losses in nearly all of the amino acids had occurred. The amino acid content of the unirradiated 26 - 50% fraction was found very similar to that of gelatin. Glucosamine was found in the 26 - 50% fraction, and hyaluronic acid was considered the most likely source.

The WDH odor from the 26 - 50% fraction could not be distilled under reduced pressure, and disappeared during attempted distillation. The WDH odor was found to disappear upon dialysis and was not found extractable by several nonaqueous solvents, including methanol. The WDH odor could be detected from three parts irradiated protein in 100,000 parts of water. The finding that the odor could be produced from a dry sample of the 26 - 50% fraction packed in helium during irradiation led to the belief that oxygen was not required for production of the WDH odor.

At the beginning of our own program, the nondialyzable fraction of hot aqueous extract of cooked beef, prepared by Hedin's method, seemed a reasonable choice of material for study. Following lyophilization, it could be dried further and irradiation in the dry state could be expected to induce long-lived unpaired spins that might be studied at leisure by ESR. The material had been well characterized and had been further purified by ammonium sulfate precipitation. After having characterized the behavior of ESR signals from the cruder material, departures from this behavior could be followed through successive purification.

Only one necessary condition for bringing about the production of WDH odor from the dry material was known--the addition of water to the sample. As expected, this was also a sufficient condition for decay of the unpaired spins, but this could not be taken as proof that the observed unpaired spins represented intermediates in the reactions leading to WDH odorants. The sole action of the water might be that of desorbing odorants that had been physically adsorbed by the material, or water could enter into a reaction resulting in WDH odorants that required no free radical intermediates. The primary task seemed that of determining whether the observed unpaired spins indeed represented free radical intermediates in the reactions giving WDH odorants.

II. EXPERIMENTAL PROCEDURES

A. Samples and Sample Preparation

The investigation was carried out for the most part upon samples prepared by Hedin's method, but samples prepared by fractionation techniques set forth by Landmann have been employed also. Samples prepared in Hedin's laboratory were used early in the investigation, and a further sample was obtained by ammonium sulfate fractionation of a Hedin material prepared in our own laboratory. This diversity of sample preparation techniques is consolidated in this section, together with observations incidental to sample preparation.

1. Two Samples Obtained from Hedin's Laboratory (QMF&CI)

The two samples received from Hedin's laboratory have been termed Samples I and II. Sample I was received in early January, 1961, and the majority of ESR work reported for this sample was performed within a few days following January 30, 1961. Sample II was received by us in Chicago on June 8, 1960, and had been stored for several months at QMF&CI prior to that date. In physical appearance, both Samples I and II resembled our own Preparations 1 and 4, being cream-colored and brittle. The ESR signal intensity from

Sample II proved approximately 1/75 that from Sample I when first received. Re-examination of Sample I immediately following this showed that it had decayed by a factor of approximately 150 during five months of storage here at room temperature.

2. Preparations of Hedin's Material in this Laboratory

Nine preparations of Hedin's crude material were carried out during the course of the investigation. The general procedure will be set forth first, followed by observations on the individual preparations.

a. General Procedure

Defatted chuck roast was obtained locally from Swift and Company and cut into one-inch cubes. A 1200 g quantity of the cubed meat was mixed with 1200 ml of distilled water in a 5000-ml round bottom boiling flask. The mixture was refluxed for 10 - 15 hours, with extensive bubble formation occurring throughout this time. The hot mixture was vacuum filtered through Whatman No. 2 paper in a Büchner funnel. The filtrate was cooled to 1 - 3°C and filtered again in the same manner. The second filtrate was concentrated from 1500 ml to 300 ml by placing in a large desiccator under vacuum at 54° ±3°C. A Welch Duo-Seal forepump protected by a dry ice-acetone trap was employed, and the process of concentration required 12 - 15 hours. This long time is attributed to a high resistance to flow between desiccator and trap.

During the first preparation, a 100-ml quantity of the concentrated filtrate was shell-frozen in dry ice-acetone and lyophilized.

The product was a brittle, golden-brown material that bonded firmly to the flask in which the process was performed. Only 150 ml was dialyzed. During succeeding preparations, however, 200 ml of concentrated filtrate was dialyzed in cellulose tubing against distilled water at 4°C. The dialysis required five days, during which the water was changed every 24 hours. A total of 1500 ml of water was used, and the last change appeared clear. The dialyzable material was discarded. The non-dialyzable fraction was shell-frozen and lyophilized, giving a soft, porous, cream-colored material that did not adhere to the vacuum flask. Lyophilization was considered complete when the material separated freely from the flask. The time for this varied from preparation to preparation.

b. Individual Preparations

Preparation 1. The beef was well marbled with fat, and was probably of commercial grade. Several tens of milliliters of fat appeared on the surface of the mixture during refluxing. The appearance of this preparation at its various stages resembled closely that of Preparation 4.

Preparation 2. The beef was extremely lean, probably utility or cutter grade. After hot filtering, only a single globule of fat approximately 5 mm in diameter appeared on the surface, and the aqueous phase was a dark orange-brown. Following concentration, the aqueous phase was easily poured at 15°C and precipitation from this phase was noted at 25°C. During dialysis there was further precipitation

of a colorless material in the tubing, which was separated by centrifugation at 300 x g at 4°C. Lyophilization of the 180 ml nondialyzable fraction required 32 hours. The lyophilized material appeared extremely hydroscopic and somewhat like a yeast cake in texture after grinding. It could be ground to a small apparent particle size easily by mortar and pestle, and it had a darker brown tint than other preparations, including Preparation 3.

Preparation 3. The beef was extremely lean, probably utility or cutter grade. After hot filtering, approximately 5 ml of fat appeared on the surface, and the aqueous phase appeared lighter in color than that of Preparation 2. Following concentration, the aqueous phase was more viscous than that of Preparation 2 at 15°C, and precipitation was noted at 25°C. During dialysis there was further precipitation of a colorless material within the tubing, which was separated by centrifugation at 300 x g at 4°C. Lyophilization of the 180 ml of nondialyzable material required 32 hours. The lyophilized material was not obviously hydroscopic, but was brittle and highly compressible. It readily acquired an electrostatic charge, and could not be ground in a mortar and pestle, therefore.

Preparation 4. The beef was well marbled with fat, and was probably of commercial grade. After hot filtering, there was approximately 75 ml of fat on the surface, and the aqueous phase was yellow-orange. Following concentration, the aqueous phase was gelatinous

at 15°C. No precipitation was observed at 25°C, and no precipitation occurred during dialysis. Lyophilization of the nondialyzable fraction required 12 hours. The lyophilized material was not obviously hydroscopic, but was brittle and highly compressible. It readily acquired an electrostatic charge, and could not be ground in a mortar and pestle, therefore.

Preparation 5. The beef was U. S. Good, well-marbled with fat. After hot filtering, 105 ml of fat appeared on the surface of the aqueous phase, and the aqueous phase was orange-yellow. Following concentration, the aqueous phase was gelatinous at 15°C. No precipitation was observed, either at 25°C or throughout dialysis. Lyophilization of the nondialyzable fraction was allowed to continue for 57 hours. In other respects, this preparation resembled Preparation 4.

Preparation 6. The beef was utility grade, very lean. After hot filtering, 10 ml of fat appeared on the surface of the aqueous phase, and the aqueous phase was yellow-brown. This was concentrated only to a 450 ml volume, so that subsequent failure to yield such a heavy precipitate as that previously obtained from lean meat is understandable. There was a slight precipitate formation during dialysis. Lyophilization of the nondialyzable fraction was considered complete after 17 hours, but parts of the preparation became a dark sirup after remaining overnight in an evacuated desiccator at room temperature. This preparation was not used.

Preparation 7. The beef was U. S. Good, well-marbled with fat. This preparation progressed as had Preparations 4 and 5. The volume of fat removed was 110 ml. The bulk of this preparation was employed in ammonium sulfate fractionation, which will be described in the following section.

Preparation 8. The beef was U. S. Good which had been carefully defatted. The volume of fat removed by filtration was 12 ml, and there was a trace of precipitate during dialysis. Otherwise, the preparation proceeded as had Preparations 4 and 5. Lyophilization was considered complete at 24 hours. Storage of the lyophilized material was in a desiccator in a nitrogen atmosphere at room temperature. Degradation of a part of this sample to a dark brown sirup has been observed.

Preparation 9. The beef was cutter grade, and was the leanest of the cuts of beef used in these preparations. Remaining portions of fat were carefully discarded before placing the meat in a flask for refluxing. No fat appeared on the surface of the hot filtrate, nor could any be removed by cold filtration. During concentration, precipitation began when the concentrate volume had reached approximately 350 ml, but concentration was continued until the volume had reached approximately 300 ml. No precipitation was observed during dialysis, perhaps because of the development of a solid phase during concentration. The nondialyzable fraction would not pour from the dialysis tubing at –

5 - 6°C because gelatinization had occurred. After lyophilization, the material resembled Preparations 5 and 8.

3. Ammonium Sulfate Fractionation of Preparation 7

A procedure given by Hedin (Ref. 1) was followed. Ten grams of Preparation 7 were dissolved in 500 ml of distilled water at 4°C to give a 2% solution. The intention was to add quantities of ammonium sulfate to give successively 25, 50, 75, and 100% saturated solutions. Unfortunately, an error in arithmetic changed these values to 21.8, 43.6, 65.4, and 87.2% of saturation. The error was compounded by failure to investigate any but the 21.8 - 43.6% fraction.

The 21.8% saturation level was reached by adding 77.0 g of ammonium sulfate to a 2% aqueous solution of beef extract at 4°C. The precipitate was allowed to form for 24 hours before centrifuging and adding an additional 77.0 g to the supernatant to give the 43.6% saturation level. The precipitate was dialyzed against water at 4°C to remove ammonium sulfate. This procedure was repeated until all of the fractions had been obtained. Nearly all of the precipitated material was in the 0 - 21.8% and the 21.9 - 43.6% fractions combined. This is in agreement with Hedin's experience that only 5% of the starting material was precipitated in the 51 - 75% and 76 - 100% fractions combined (Ref. 1).

4. A Preparation Following Landmann's Procedure (Ref. 6)

U. S. Good grade beef chuck was obtained fresh locally from Swift and Company and ground in a meat grinder with some fat

being rejected. A kilogram of the ground meat was extracted with distilled water until very little pink color remained in the residue. Eleven liters of water in one- and two-liter aliquots were required to effect this. The lyophilized residue was extracted in Soxhlet extractors with hexane. The hexane was removed from the residue under vacuum. The dried fat-free residue from this first preparation weighed 132.5 g. This material was divided into three portions and each portion was extracted with a liter of methanol divided into several aliquots. The methanol was removed and the residue was dried under vacuum. The yield of methanol-extractable material was approximately 4 grams. A two-gram sample of the methanol extract was dissolved in 25 ml of chloroform and applied to the chromatography column. The chromatographic procedure was as follows:

A slurry of dried silicic acid (Mallinckrodt chromatographic grade, 100 mesh) in chloroform was poured into the chromatographic tube which was fitted with a coarse grade sintered glass disc. The silicic acid was treated to remove some fines before use to insure uniform flow. Chloroform was allowed to flow through the column for some time before the sample was introduced and again after introduction of the sample. Methanol was then fed into the chloroform reservoir, which was an aspirator bottle containing two liters of chloroform. This was mixed throughout the run with a magnetic stirrer. Flow rates were adjusted so that the methanol flowed into the chloroform at the

same rate the mixture flowed into the column, which was at the same rate as the elution. The column used was a 5 x 50 cm column. The automatic fraction collector used was operated on a time basis, and fraction volumes were about 25 ml. The column appearance was similar to that described by Landmann. There were two yellow bands which passed down the column and additional material which remained at the top. The second band, which was broader than the first, did not divide into a double band, however, which was probably due to a shorter elution time. The samples were dried with dry air and vacuum. Twenty-seven fractions were collected.

An additional preparation proceeded to the chromatography stage only.

B. Sample Irradiation

The Co^{60} sources used in this investigation were calibrated on September 16, 1960 through the medium of Bausch and Lomb glass chips against a standard Co^{60} source owned by Convair. The dose rate from the Convair source was known in terms of roentgen/hr (r/hr). In reporting absorbed dose in rad, a conversion factor of 93 ergs/gm-roentgen was assumed. The most-used source had the configuration of a hollow cylinder in which the dose rate was considered homogeneous throughout a cylindrical volume measuring 1/2" in diameter by 3" in length. The samples lay within this volume. The calibrated dose rate on September 16, 1960, was 2.13×10^5 r/hr. Irradiation times were chosen

such that all samples received not less than 4.6×10^6 nor more than 4.8×10^6 rad.

Prior to irradiation, samples were vacuum dried to constant weight at 40°C following powdering in an Osterizer, but powdering of Preparation 1 was accomplished by passing it through progressively finer nickel chromium screens, the finest being 50 mesh. Preparations other than Preparation 1 were sealed in an evacuated Pyrex tube for irradiation. Preparation 1 was sealed in dry air. Preparation 9 was irradiated both in air and in vacuum.

C. ESR Instrumentation

A Varian V-4500-10A ESR spectrometer with 100 kilocycle-per-second modulation operating at 9500 megacycles per second has been used. The polarizing magnetic field of 3380 gauss has been supplied by a Varian V-4012A 12-inch magnet and associated power supply. The cavity was a Varian V-4531 which has a window permitting irradiation of the sample in the visible or ultraviolet or (somewhat less conveniently) by x-rays simultaneously with ESR observation. Spectroscopic splitting factors (g-values) and line widths were determined by placing a dilute solution of peroxylaminedisulfonate negative ion in a capillary tube near the sample. This ion gives three lines of equal amplitude separated by 13.0 gauss with a g-value of 2.00550 ±0.00005 (Varian EPR at Work Series, No. 28). Signal intensities were determined by double numerical integration of the signal. This was translated into a measure of unpaired

spin population in the sample by comparing the signal intensity of the sample with that obtained from coal samples with known spin populations. These in turn had been compared with signal intensities from a $CuSO_4 \cdot 5H_2O$ single crystal of known mass, and with calibrated samples of pitch supplied by Varian Associates. Accuracy of spin population measurements is not considered better than ±100%, however.

D. Odor Analysis

Odor observations were performed by the investigators and by an informal panel when the occasion seemed to demand. Initially, the members of the panel did not find it at all obvious to characterize odor from moistened irradiated Hedin material as similar to that arising from wet dog hair, but recognition of it in these terms was learned rapidly. Throughout the program, all members of the panel were considered capable of determining the presence or absence of WDH odor. In addition, the investigators were frequently interested in comparing the intensity of odor from a number of samples. To determine the panel's capabilities for quantitative odor determination, different quantities of irradiated beef extract samples (Hedin's crude material) were suspended in 1.0 ml of distilled water and presented for analysis in 5 ml beakers. The beakers were presented in pairs with the question, "From which sample is the odor the most intense?" The panel could not distinguish differences among quantities when the quantities were less than 10 mg. Quantities of less than 5 mg could not be detected with certainty. Quantities

differing by 40 mg over the range from 10 mg to at least 100 mg were distinguished with certainty, but 20 mg differences could not be distinguished. These limitations must be considered in evaluating the meaningfulness of the investigations reported here. Surely difficulty in quantitative odor determination is the greatest barrier to characterization of the odor and establishment of its source. A useful olfactometer for comparing the intensity of odor from an unknown sample with that from a standard sample might prove to be the answer.

Samples for odor analysis were suspended in 1.0 ml of distilled water in a vial or 5 ml beaker, and a control was presented for comparison. The relationships "greater than," "less than," and "equal to" had meaning, but more narrowly defined relationships did not.

III. PRESENTATION OF DATA AND DISCUSSION OF RESULTS

A. Characterization of ESR Signals

Figures 1 - 9 are ESR signals from irradiated but otherwise untreated preparations. They fall within two categories. In the first category are the ESR signals from Preparations 1 and 4, shown in Figures 1 and 2, which show clearly resolved structure in the high field half of the signal. In the second category fall the remaining signals, which have an asymmetry which broadens and reduces the amplitude of the high field half of the signal with respect to the low field half. The only apparent exception to the general appearance arose from Preparation 3, in which the asymmetry has seemed to reverse the relative amplitudes of the two halves of the signal. Efforts to obtain a signal in the first category from a preparation following Preparation 4 were in vain. After Preparations 1 - 4 had been investigated, the quantity of fat given up in filtration seemed to correlate with the appearance of the ESR signal; but later high fat preparations did not give signals similar to those from Preparations 1 and 4. Consequently, the second category signal shape is believed the more general one. Odor analysis indicated no differences relating to signal shape.

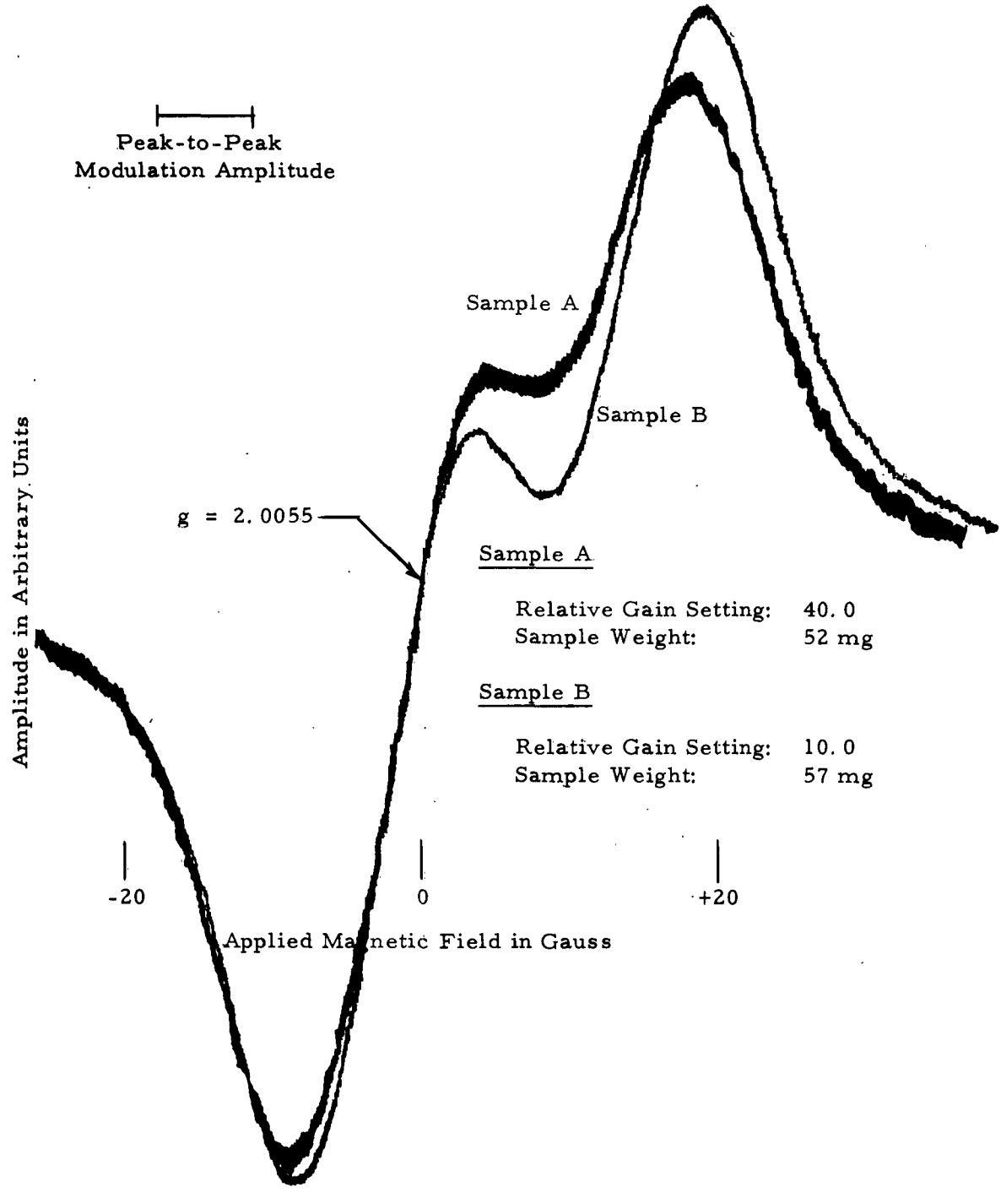


FIGURE 1. ESR SIGNALS FROM FREE RADICALS IN PREPARATION 1, SAMPLES A AND B

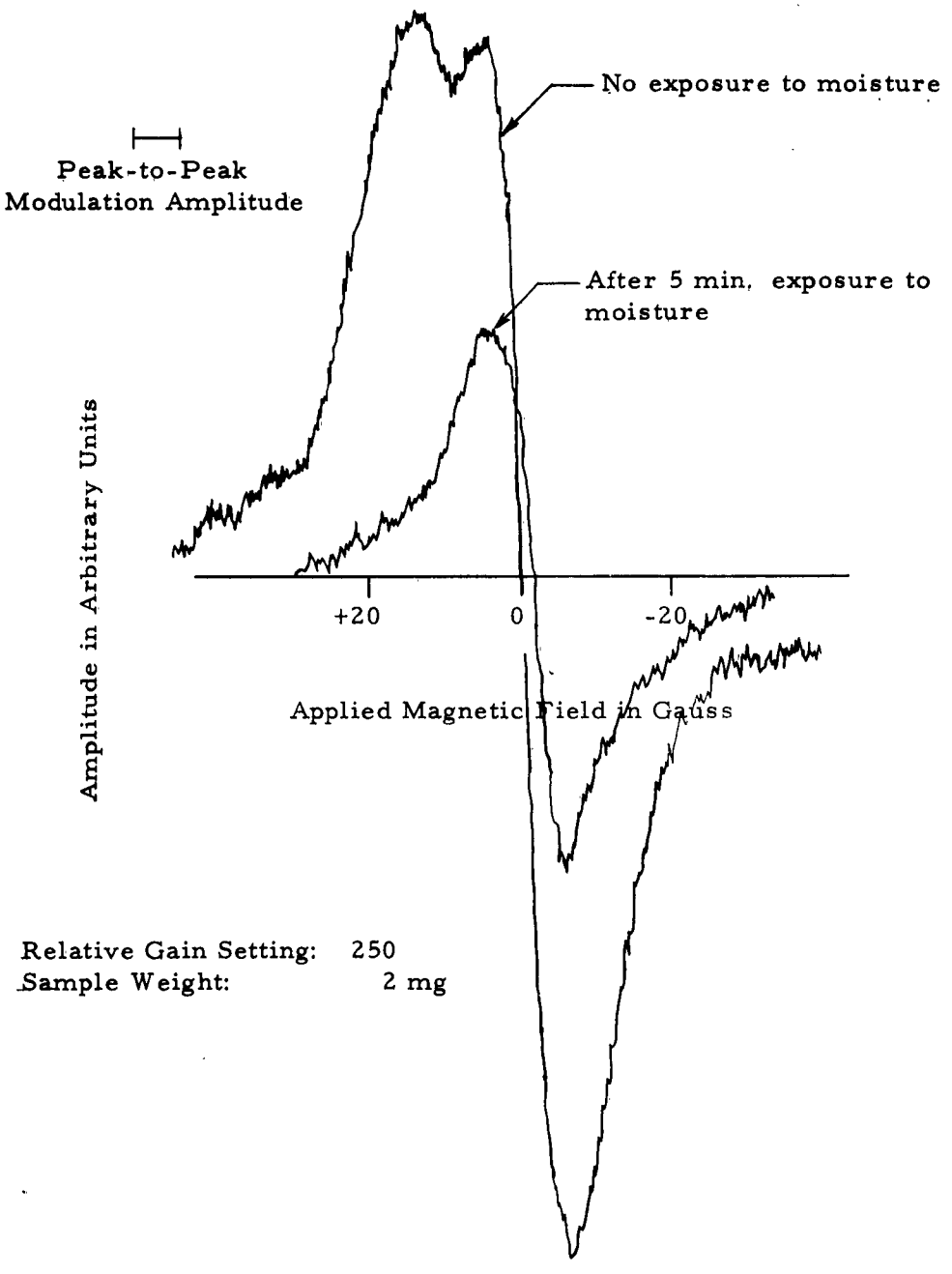


FIGURE 2. CHANGE IN SHAPE OF ESR SIGNAL FROM PREPARATION 4 DURING FIRST FIVE MINUTES OF EXPOSURE TO MOISTURE

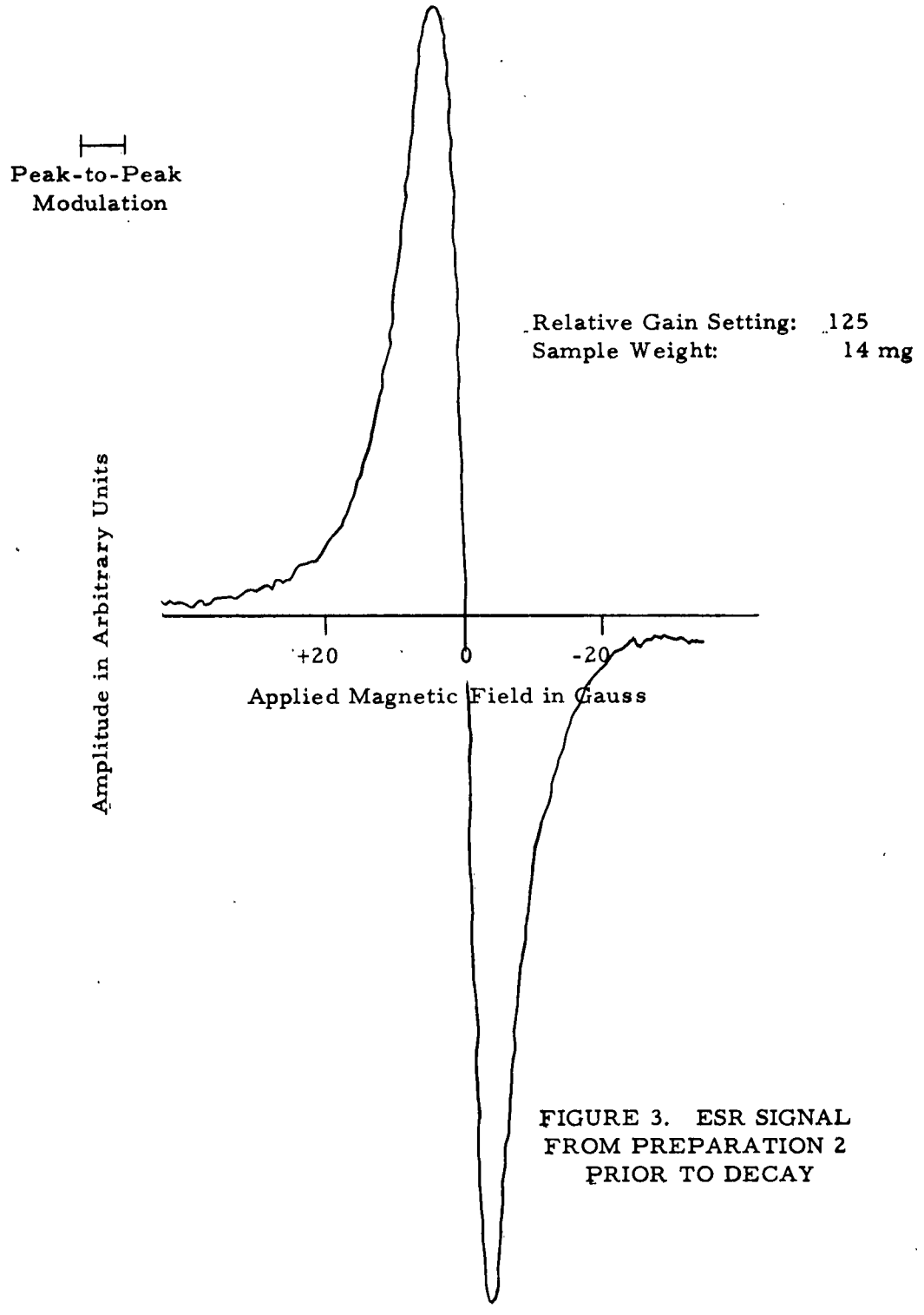


FIGURE 3. ESR SIGNAL FROM PREPARATION 2 PRIOR TO DECAY

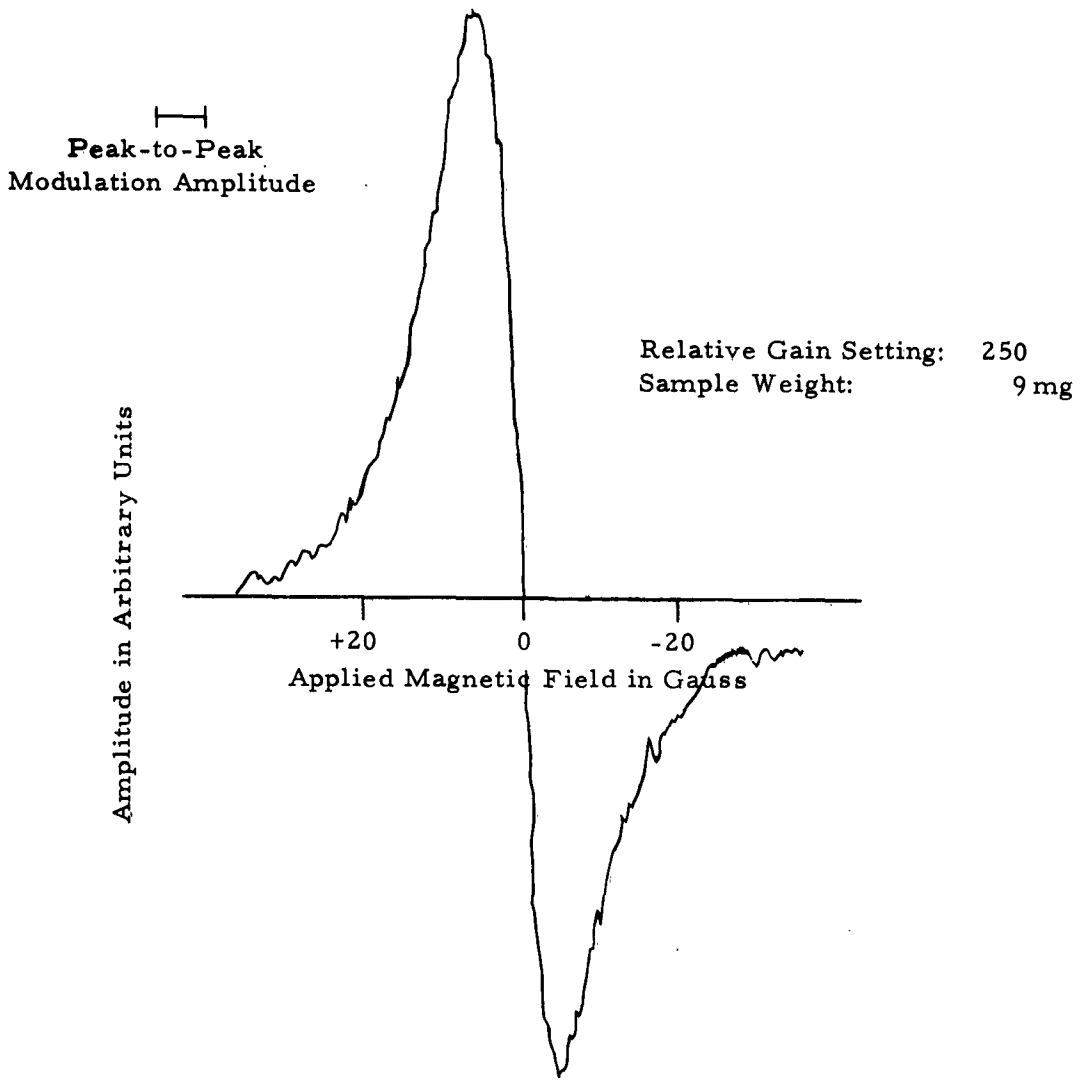


FIGURE 4, ESR SIGNAL FROM PREPARATION 3 PRIOR TO DECAY

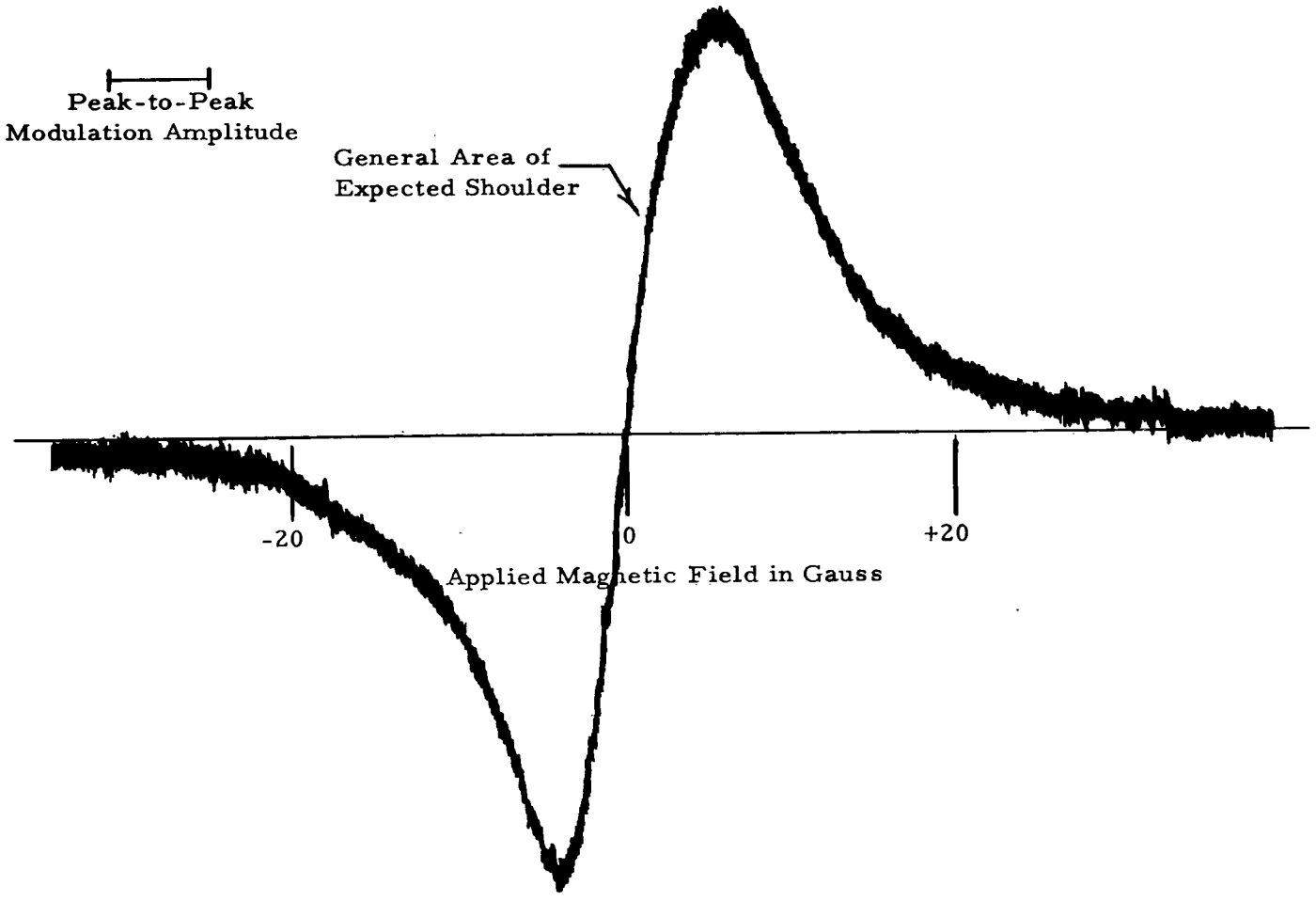


FIGURE 5. ESR SPECTRUM OF IRRADIATED SAMPLE OF PREPARATION 5

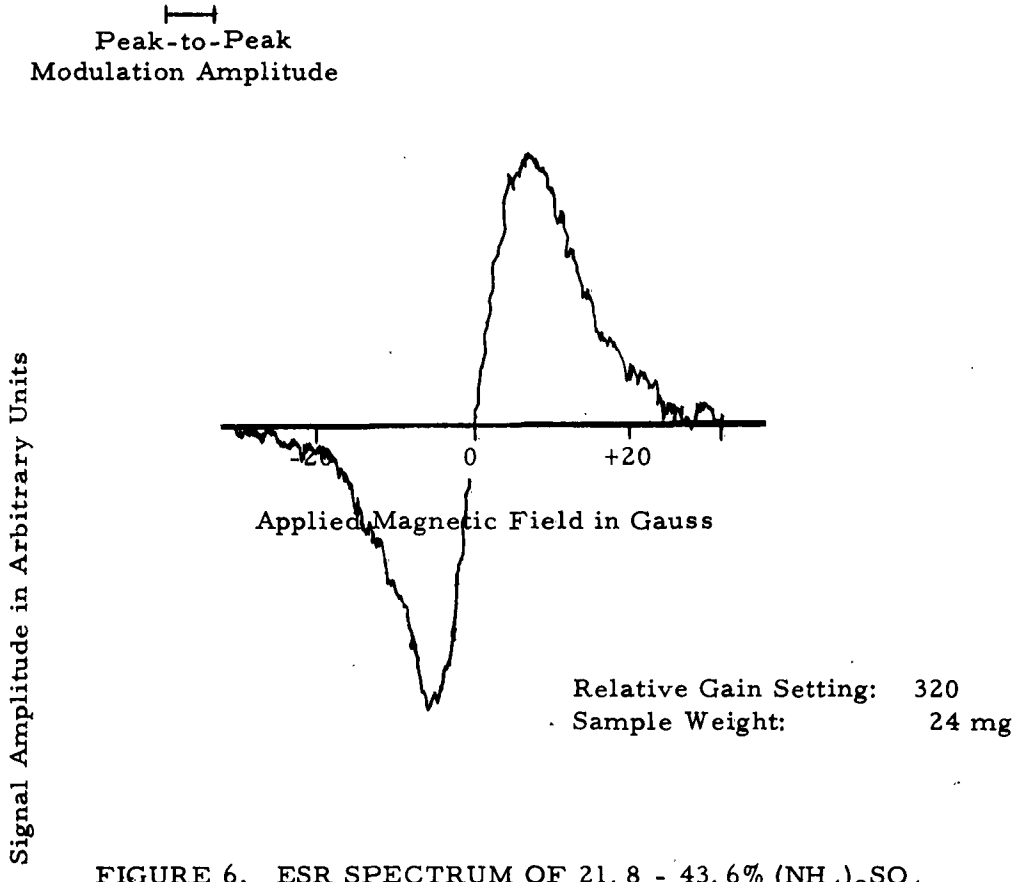


FIGURE 6. ESR SPECTRUM OF 21.8 - 43.6% $(NH_4)_2SO_4$ PRECIPITATION FRACTION FROM PREPARATION 7

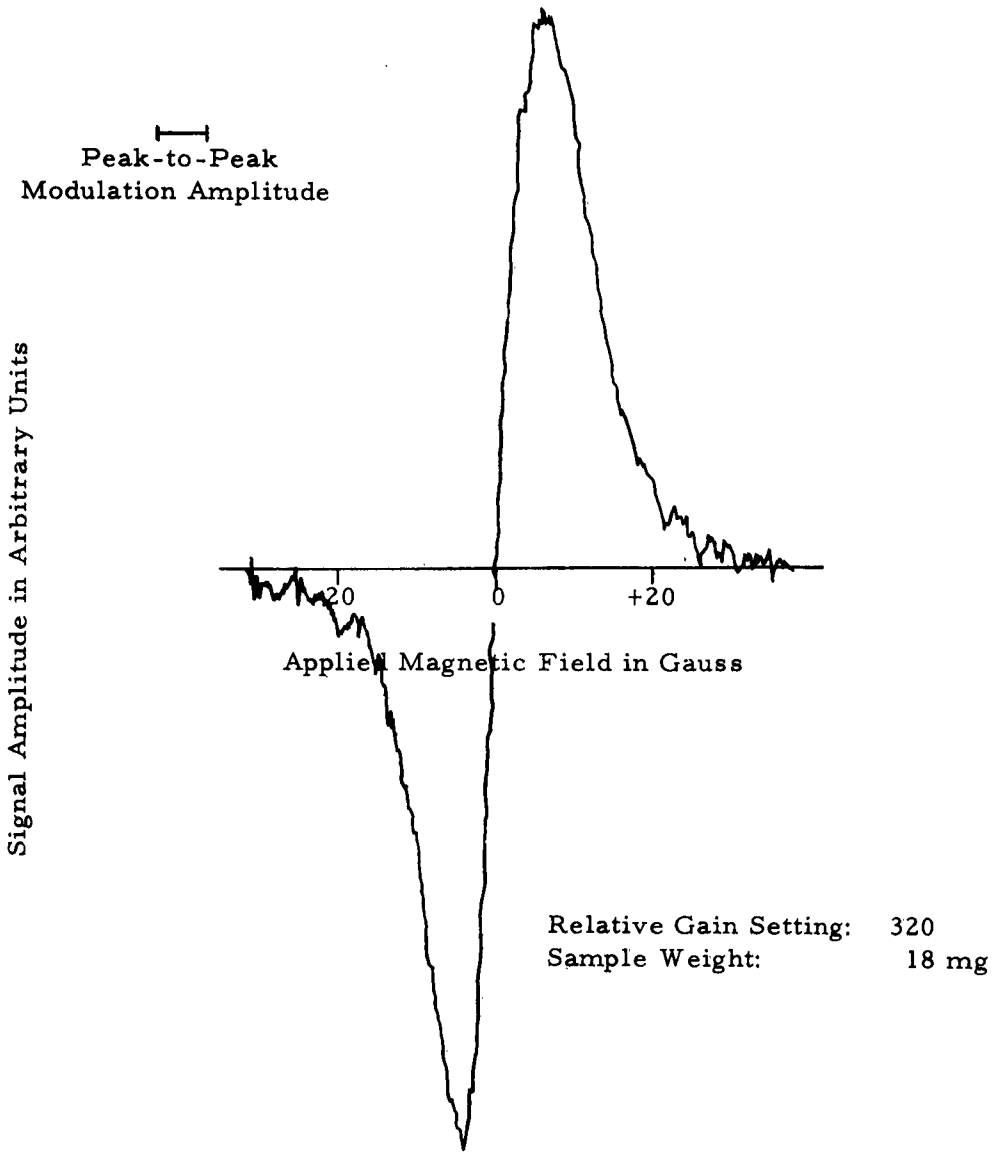


FIGURE 7. ESR SPECTRUM OF PREPARATION 8

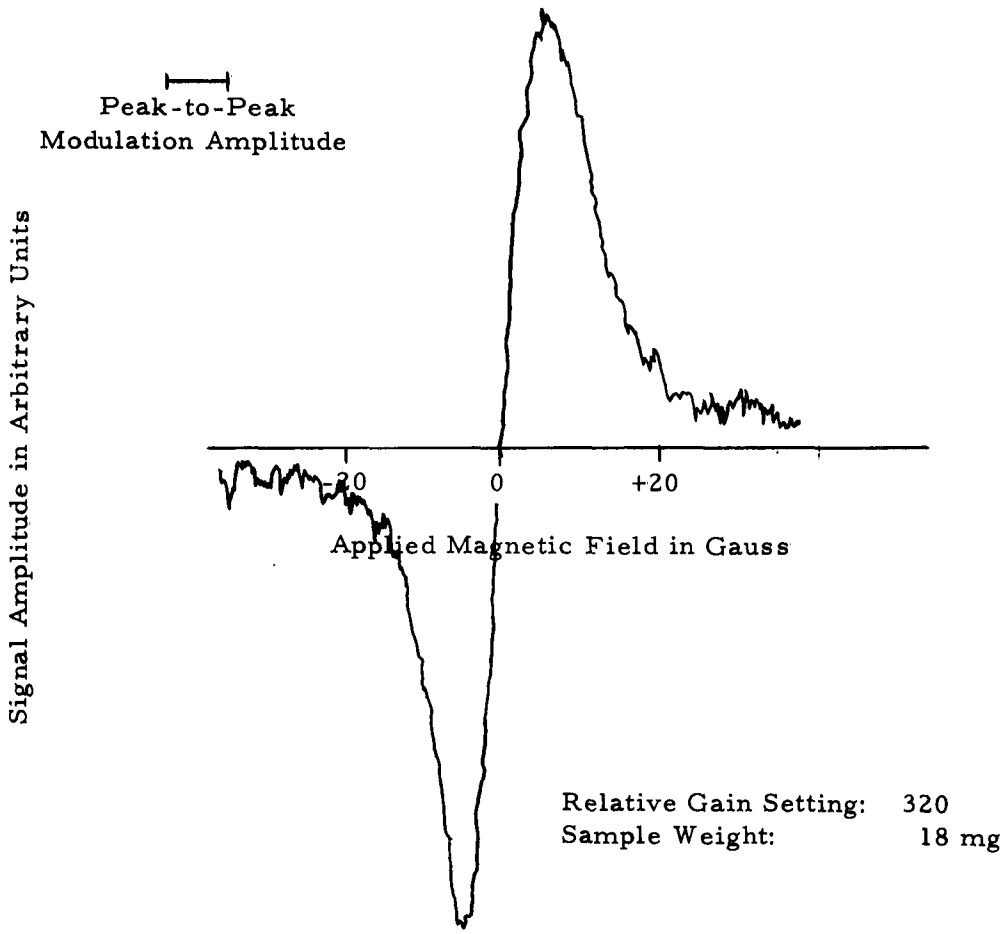


FIGURE 8. ESR SPECTRUM OF PREPARATION 9

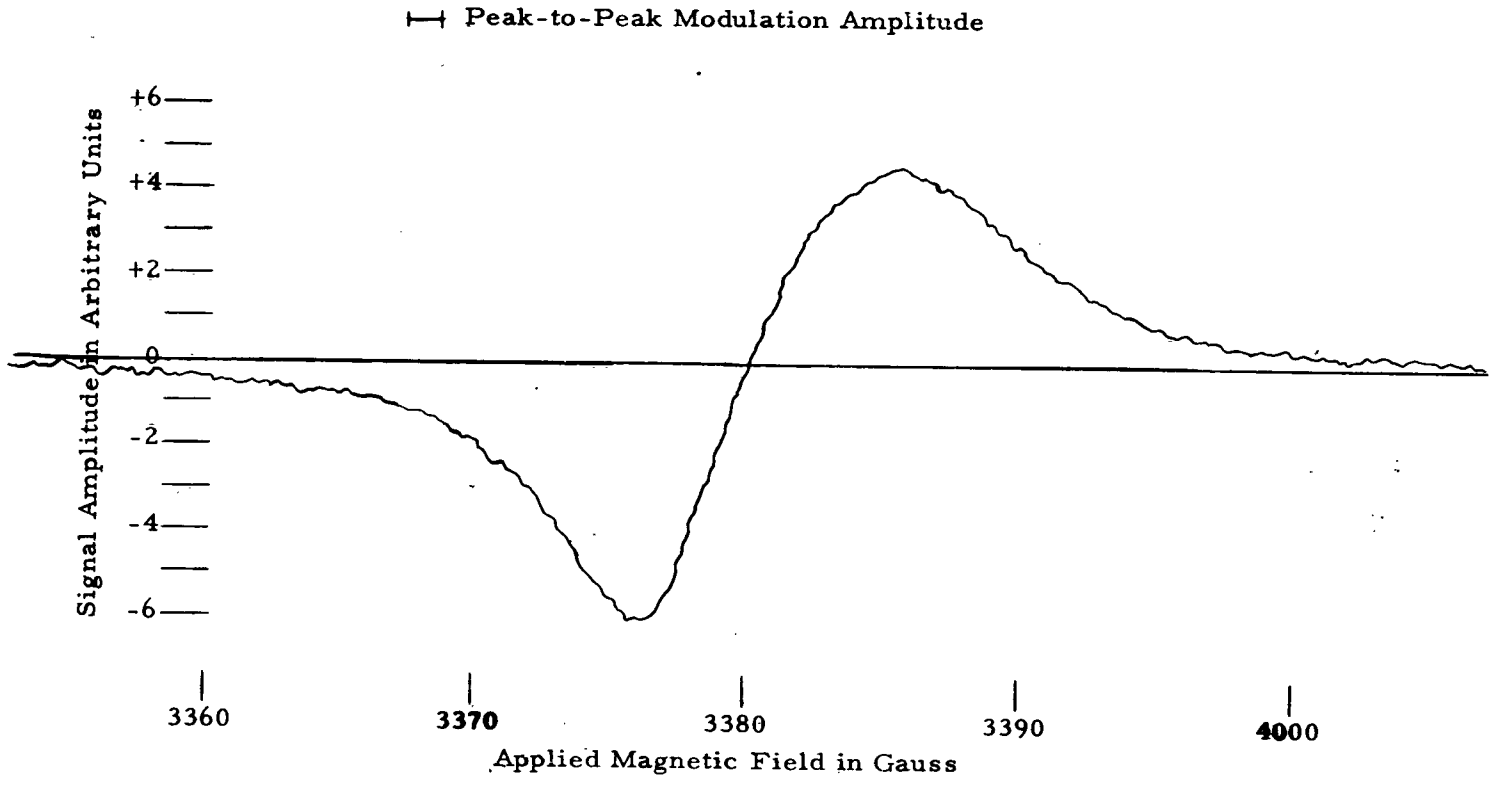
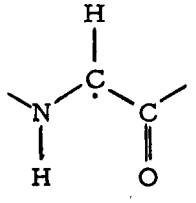


FIGURE 9. ESR SIGNAL FROM SAMPLE I WHEN FIRST RECEIVED

Analysis of the signal shape from a sample of Preparation 1 has shown that it can be considered either as an asymmetrical doublet with a separation of 12 gauss or as the superposition of two signals, the high field member having the lower intensity relative to the principal or low field member. Assuming the shapes of both signals symmetrical, the g values of the two signals were 2.007 and 1.998, and their intensity ratio was 1.4/1. Of the two possibilities, the doublet structure seems the better. Hedin has found in material prepared by his method 5% nucleoprotein, with the remainder being protein containing 2.05×10^{-4} moles/gm of glycine, 0.35×10^{-4} moles/gm of cysteic acid, 0.75×10^{-4} moles/gm of methionine, and 0.80×10^{-4} moles/gm of cysteine (Ref. 3). Moore and Stein (Ref. 7) report as cystine that fraction identified as cysteine by Hedin. Irradiation of dry proteins containing glycine and either cystine or cysteine results in ESR signals characteristic of the glycine content (glycyl signals) and/or the cysteine plus cystine content (cystinyl signals). Cystinyl signals disappear when the sample is exposed to oxygen, but the glycyl signal is found stable to oxygen exposure (Ref. 8). The glycyl signal is a doublet with a separation of 12 gauss (Ref. 9). Denaturation of the protein prior to irradiation has been found to favor the induction of the glycyl signal, whereas native protein gives cystinyl signals preferentially (Ref. 10). Our own samples have been exposed to oxygen prior to observation. As a consequence of this and of its apparent doublet structure, the ESR signal

from Preparation 1 is probably due mainly to free radicals at glycyl structures in the material, and that from Preparation 4 is at least partly so.

There are other structures within the material that might be stable in oxygen. Glycyl-DL-methionine gives an oxygen-stable structure that has been considered $X-S-CH_2-O\overset{\cdot\cdot}{-}\overset{\cdot\cdot}{O}$ (Ref. 11), although peroxides are, in general, observed to decay rapidly. Methionine alone gives a radical which decays rapidly in air. Consequently, some methionine in protein might give a stable radical. The more general type of ESR signal given by Hedin's crude material may thus be a superposition on a glycyl signal of a signal from an unpaired electron on a sulfur-containing amino acid side chain, the effect being to destroy the resolution of the glycyl doublet. Other possibilities are that the whole signal is due to a different radical derived from the glycyl radical

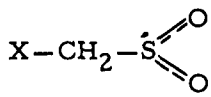


or that it is due altogether to an unpaired electron associated with sulfur-oxygen. However, the integral of the high field half of the ESR signal is generally larger than that of the low field half. This is taken to mean that there exists an unresolved signal in the high field half of

the observed ESR signal. This is consistent with the viewpoint that the glycyl doublet is present but is being obscured by some other signal.

There is a suggestive ESR signal that arises from the dithionite ion $(O_2SSO_2)^=$ (Ref. 12). The signal from a sample of polycrystalline sodium hydrosulfite $Na_2S_2O_4 \cdot 2H_2O$ superimposes almost precisely upon the signal from Preparation 9, with an equal g value, provided that the direction of increasing magnetic field of one is reversed with respect to that of the other. The free radical, which may be the reducing agent $(\dot{S}O_2)^-$, is stable, surviving heating to 60 - 70°C. In the dry form it is resistant to destruction by oxygen, from our own observation; but in aqueous solution it is quickly destroyed by oxygen, giving rise to a precipitate of free sulfur presumably. Sodium hydrosulfite and Hedin's crude unirradiated material were dissolved together in oxygen-free water, and the water was evaporated under vacuum. The resulting dry material displayed a strong ESR signal with a line width somewhat reduced from that of the polycrystalline sodium hydrosulfite. A few grains of the powdered sodium hydrosulfite in an aqueous suspension of Hedin's crude material gave rise to a strong odor which resembled wet chicken feathers more than wet dog hair (but which some of the panel members insisted was wet dog hair nonetheless). If the sense of asymmetry of the ESR signal from sodium hydrosulfite were not reversed from that from the irradiated meat extract, the possibility that $(\dot{S}O_2)^-$ radicals are the source of the ESR signal of the more general

line shape would be worth exploring in some detail. To rule out the possible existence of these radicals on the basis of signal asymmetry alone would be unfair, if the asymmetry is due to the superposition of two signals. Perhaps the radical



that could result from the loss of a proton from cysteic acid, would give an ESR signal of precise fit. No ESR work on cysteic acid could be found.

Yet one more possibility should be mentioned. Boag and Müller (Ref. 13) have reported a radiation-induced ESR signal from deoxyribonucleic acid that in the dry state was long-lived in air at room temperature and which, although it was not well characterized, bears a superficial resemblance to the more general ESR signal shape from the meat extract. Energy transfer from molecule to molecule in molecular mixes such as the meat extracts, resulting in radicals appearing in molecules that have not absorbed energy directly from ionizing radiation, is well known (Ref. 14). By this mechanism radicals might appear in nucleic acids in the extract with reasonably high quantum yield even though the nucleic acid content of the extract is low.

Free radical densities in several sample preparations were measured and the results are given in Table 1. The free radical density

TABLE 1. FREE RADICAL DENSITIES FROM SEVERAL PREPARATIONS

	Free Radical Density	
Sample	Spins/Gram	Moles/Gram
I	1.3×10^{17}	2×10^{-7}
2	3.5×10^{17}	6×10^{-7}
3	3.5×10^{17}	6×10^{-7}
4	3.3×10^{18}	5×10^{-6}

in all samples is lower by at least an order of magnitude than any of the reported amino acid concentrations with which the free radicals might be associated. The rapidity of the free radical decay during the first few minutes following irradiation is not known, nor would the knowledge be very meaningful considering the long irradiation times. There is, however, evidence that the hourly decay rate of free radicals during storage in vacuum at 27°C is high immediately following irradiation. Two samples from Preparation 1, irradiated simultaneously, were opened and examined by ESR at 5 hours (Sample B) and at 28 hours (Sample A) following the end of irradiation. The free radical density of Sample A was lower than that of Sample B by a factor of 3.5. Furthermore, Sample A had somewhat less doublet character than Sample B. Consequently, the measured free radical densities were considerably lower than those that would have been measured had the dose rate been higher and had the observations been made within the first few minutes following irradiation without allowing oxygen to contact the sample. The free radical yield might have been even higher had irradiations at liquid nitrogen temperature been performed. Consequently, there seems little to be learned by comparing the free radical densities with concentrations of any of the known constituents of the sample, for such a comparison rules out association of the free radicals with none of them.

B. Free Radical Decay Studies

1. Decay with Addition of Moisture

This investigation will be considered in two parts. In one part bulk samples from Preparation 1 were exposed to high humidity at a known temperature for three different time intervals in a constant humidity chamber, and both ESR signal decay and odor production were observed by sampling these. In the second investigation, ESR signal decay was observed in greater detail by passing tank nitrogen saturated with water vapor through the sample in the microwave cavity. An attempt was made to trap odorants released.

a. Exposure of Samples in Constant Humidity Chamber

Samples for ESR investigation were placed in 4 mm OD by 3 mm ID quartz tubes filled to a height of 23 mm. The sample height was chosen to coincide with the long dimension of the cavity cross section. The effective time constant of the instrument was 10^{-4} sec. Peak-to-peak modulation amplitude was 6 gauss. Klystron frequency was 9500 megacycles per second at a magnetic field of 3380 gauss.

In Figure 1, ESR signals from two irradiated but otherwise untreated samples from Preparation 1 are shown superimposed. The difference between the two has already been discussed in Section III. A. The material from which Sample A was taken (Material A)

had been stored for 28 hours prior to ESR observation, whereas the source of Sample B (Material B) had been stored for only 5 hours.

The decay behavior of Material A only was followed. The sampling procedure employed for obtaining the data given in Table 2 was as follows:

After Material A had been irradiated, a portion of it was maintained dry and an ESR signal was obtained from that portion. A portion of the remainder was exposed in the humidity chamber for one minute and this was examined by ESR. A second portion of the remainder was exposed to high humidity for 10 minutes and a portion of this was examined by ESR. That part of the sample which had been exposed to high humidity for 10 minutes was redried and re-irradiated to a dose of 4.6×10^6 rad, and a portion of it was examined by ESR. The signal from this sample is shown in Figure 10. The free radical intensity obtained from this sample is less than that first obtained from the dry Sample A, and much less than that obtained from the dry Sample B. The doublet character of the signal has remained, showing that destruction of the free radical content of the sample is not necessarily accompanied by a change from this line shape to the more general line shape.

Figure 11 is an ESR signal taken under the same instrumental conditions as those used for obtaining signals from the dry irradiated but otherwise untreated samples. The sample was taken from Material A which had been exposed for one minute to a 98% relative

TABLE 2. RELATIVE INTENSITIES OF ESR SIGNALS FROM RADIATION-INDUCED FREE RADICALS IN PREPARATION 1 AS A FUNCTION OF DURATION OF EXPOSURE TO 98% RELATIVE HUMIDITY AT 27°C

Duration of Exposure (Min.)	Uncorrected ESR Signal Intensity (Arbitrary Units)		Coal Signal Amplitude (Chart Divisions)		Sample Weight (mg)		Corrected Signal Intensity (Arbitrary Units)	
	Material		Material		Material		Material	
	A	B	A	B	A	B	A	B
0	6460*	25400**	171*	173**	52*	57**	7.3*	25.8**
1	2590	Not Meas.	170		51		3.0	
10	0	Not Meas.	170		56		0	
Redried & Re-Irradiated	6680	Not Meas.	173		73		5.3	

*Sample A

**Sample B

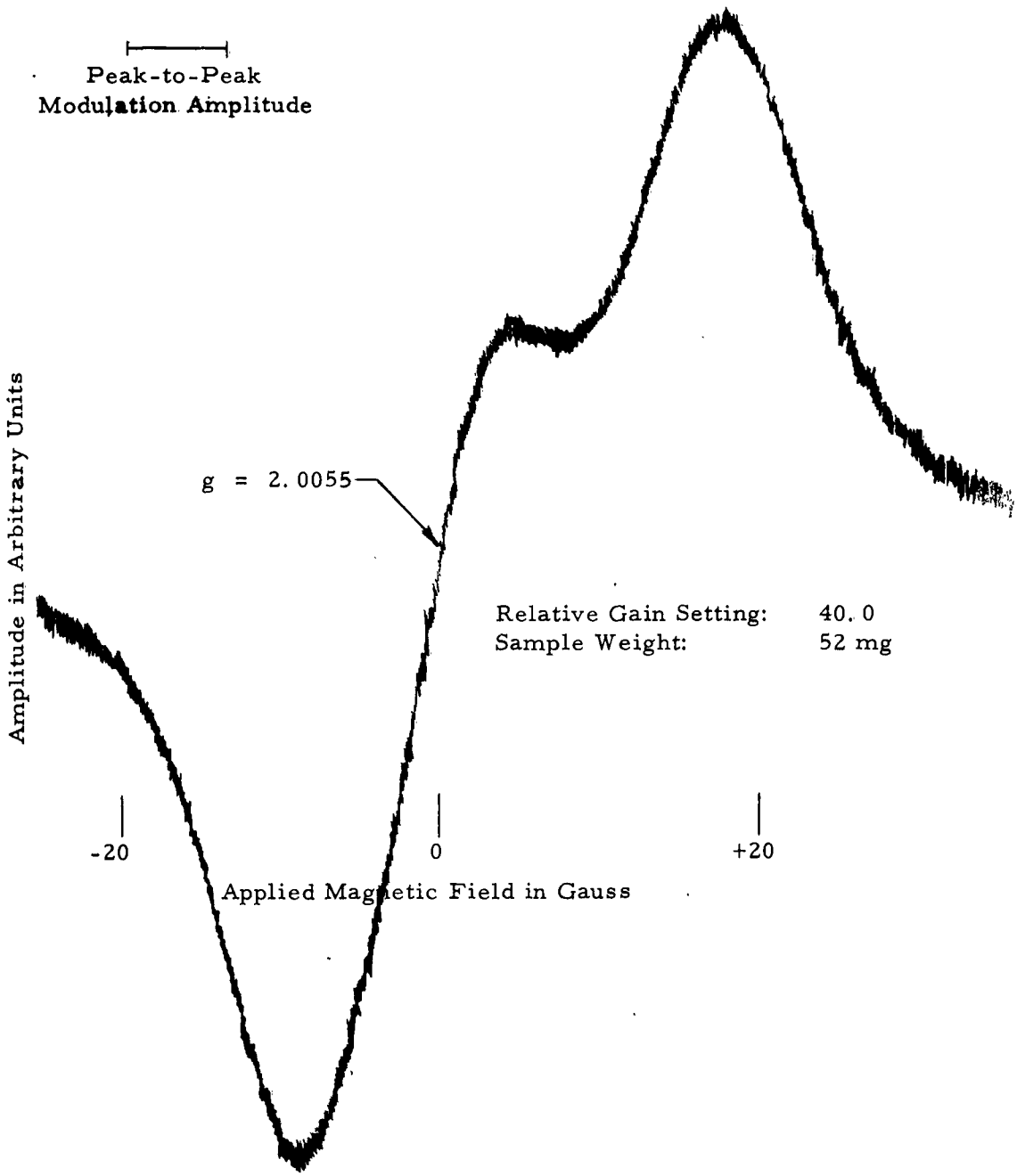


FIGURE 10. ESR SIGNAL FROM THE DRY RE-IRRADIATED SAMPLE OF MATERIAL A, PREPARATION 1

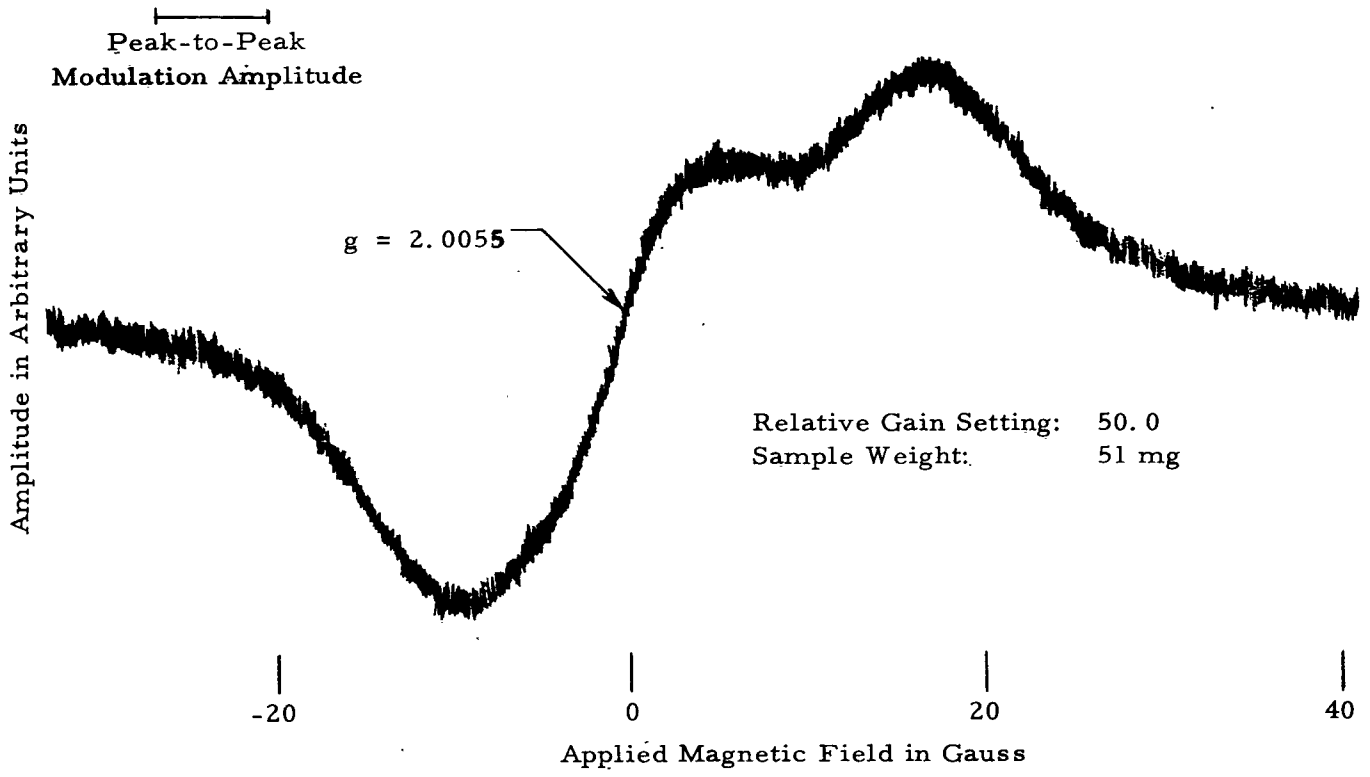


FIGURE 11. ESR SIGNAL FROM IRRADIATED MATERIAL A, PREPARATION I
AFTER ONE MINUTE EXPOSURE TO 98% RELATIVE HUMIDITY AT 27°C

humidity at 27°C. The decrease in signal amplitude and relative constancy of the line shape are the noteworthy features. Table 2 gives several ESR signal intensity measurements obtained by twice integrating the first derivative signals under discussion. Instrument gain was determined by comparison with a coal standard. Sample weights were not corrected for moisture content. The corrected signal intensities were obtained according to the formula:

$$\frac{\text{Uncorrected Signal Intensity}}{\text{Coal Signal Amplitude x Sample Weight}} = \text{Corrected Intensity in Arbitrary Units}$$

Two samples of equal weight of the dry irradiated extract were placed in small weighing bottles, and the odors from each were compared by an informal panel. An odor was detected, in general, from both bottles, This was not unpleasant and bore a resemblance to bacon, according to some members of the panel. The bottles were then subjected to steps of 1, 10, and 30 minutes' exposure to 98% relative humidity at 27°C, with one bottle always lagging one step behind the other bottle for comparison. For example, in the first exposure to high humidity one bottle was exposed for one minute while the other bottle was left dry. Upon exposure to moisture, the intensity of odor was found to increase. However, the bottle with 30 minutes' exposure gave less odor than that exposed for 10 minutes. This was taken as an indicated correlation between odor production and the ESR observations. As the quantity of odor increased, the quality became increasingly

unpleasant, but no one described it in terms of wet dog hair. This was early in the program, and the panel shortly learned to describe odors in terms of wet dog hair. In view of later odor analyses made by the same informal panel, the abilities of its members to distinguish between the several sorts of odors encountered in the program must be questioned.

b. Exposure of Samples to Saturated Nitrogen in the Microwave Cavity

Samples were prepared by packing 4 mm OD by 3 mm ID quartz tubes to a length of 1 cm at the center of the tube, enclosing these small samples between plugs of glass wool. Weights of the samples were obtained by difference. The preparation of these samples for ESR investigation was carried out immediately prior to the observation. Nitrogen gas was passed through a bubbler in water at 25°C, and then passed through the sample at the rate of 20 cc/minute. An attempt was made to trap odorants from the exhaust gas in a liquid nitrogen trap. The points on the decay curves (Fig. 12) were obtained from samples from Preparations 2 and 3 simply by measuring the peak-to-peak amplitude of the signal, since line width and line shape remained constant. Comparison of the initial signal intensities from the samples of Preparation 4 with the first complete signal drawn out in the decay study was performed by numerical integration because of a change in signal shape. This change in signal shape is shown in Figure 2, the low intensity signal having been taken following the first 5 minutes of

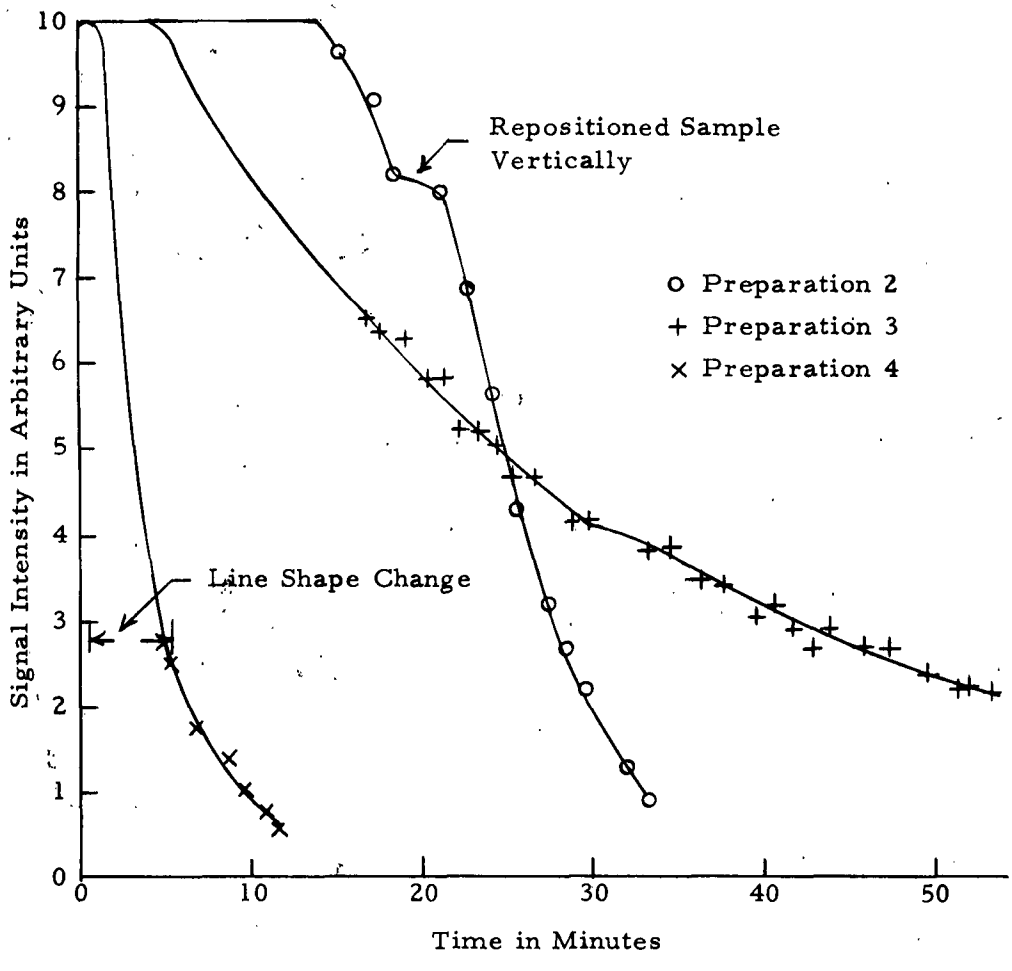


FIGURE 12. FREE RADICAL DECAY CURVES FOR PREPARATIONS 2, 3, AND 4 EXPOSED TO NITROGEN SATURATED WITH WATER VAPOR

decay. In the case of the sample from Preparation 4 also, the peak-to-peak amplitude measured had to be corrected for a decrease in line width which amounted to approximately 14% during the last 7 minutes of decay. The difference between samples from Preparations 2 and 4 appears to lie in the lengths of time between the introduction of moisture and the onset of decay. The behavior of the sample from Preparation 3 is not comparable.

The samples from Preparations 3 and 4 did not change in physical appearance during the free radical decay, but the sample from Preparation 2 changed radically. Figure 13 shows how the sample shrank, beginning at the top of the sample where the moisture was introduced and spreading to the bottom of the sample. It was as if the upper part of the sample absorbed moisture from the stream totally until saturated before allowing moisture to enter the lower parts of the sample. Linear shrinkage was estimated at 50% during decay. One could see the dark brown zone heavily laden with moisture spreading down from the top of the sample, and this spread seemed to parallel the decay of the free radicals. The break in the decay curve from the sample from Preparation 2 at 20 minutes is explained by a vertical repositioning of the sample which put the free radicals into a more sensitive part of the cavity. The apparent break in the curve from the sample from Preparation 3 at about 32 minutes is unexplained. The decay data were normalized to the same initial intensity for presentation.

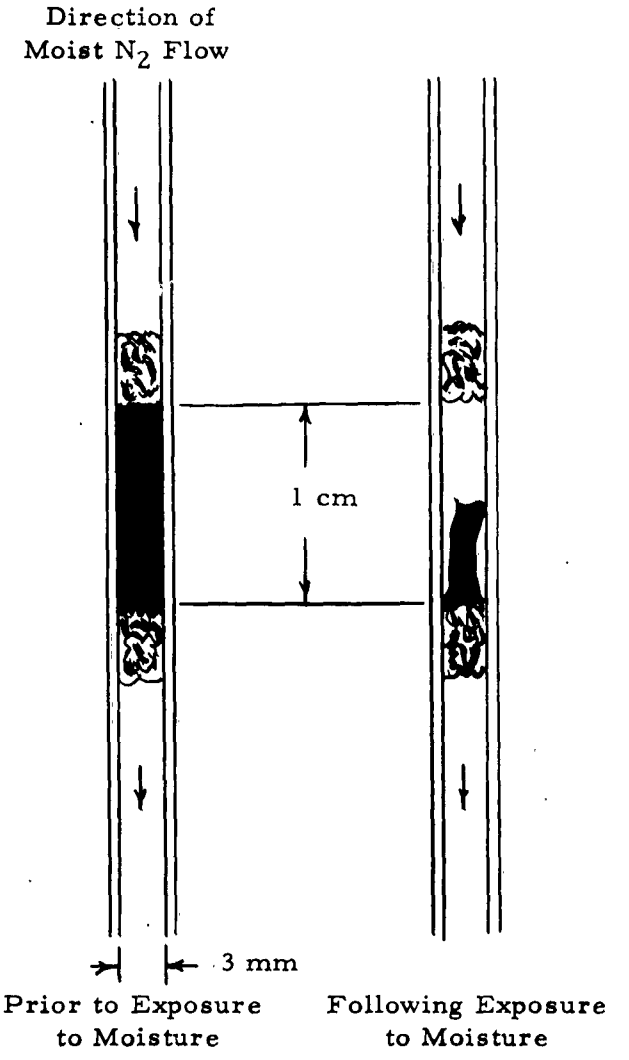


FIGURE 13. SHRINKAGE OF PREPARATION 2 DURING EXPOSURE TO MOISTURE

There was no conclusive detection of WDH odor in the traps, which is not surprising in view of the sample sizes. The samples from Preparations 2, 3, and 4 weighed 14, 9, and 2 milligrams, respectively.

2. Decay at Varying Temperatures

a. ESR Signal Decay at 60 and 100°C Using Preparation 5

Samples from Preparation 5 were taken for determining elevated temperature effects on ESR behavior. Samples were exposed to air during heating. Two temperatures were employed. At 60°C ±2°, it was observed that the ESR signal intensity of the samples was only moderately influenced; and beyond two hours' exposure, no further decay could be induced by this temperature. The total decay pattern is given by Table 3. The study was initiated in triplicate, but it became necessary to discard one of these because of ESR signal interference from the sample tube.

The second temperature employed yielded marked effects. At 100°C ±2°, the ESR response steadily decreased as the time of exposure increased up to 115 hours. Beyond 115 hours, exposure of samples to 100°C failed to increase the free radical decay. Table 4 tabulates these results. Throughout the decay study instrument settings remained unchanged; and instrument sensitivity, determined by a coal standard, remained very nearly constant over the period of investigation. A control sample taken from the same preparation as those used in the

TABLE 3. DECAY OF ESR SIGNAL INTENSITY AT 60°C

Sample	Time at 60°C	Cumulative Signal Decay in %
1	10 min	5
2	10 min	0
1	20 min	5
2	20 min	0
1	1 hour	12
2	1 hour	8
1	2 hours	18
2	2 hours	14

TABLE 4. DECAY OF ESR SIGNAL INTENSITY AT 100°C

Sample	Time at 100°C	Cumulative Signal Decay in %
1	5 min	20
2	"	24.5
3	"	30.5
1	10 min	25
2	"	30
3	"	34.5
1	30 min	38
2	"	40
3	"	44.5
1	2 hours	48.5
2	"	50.5
3	"	54
1	4 hours	54
2	"	57
3	"	58
1	18 hours	70
2	"	70
3	"	70
1	66 hours	81
2	"	82.5
3	"	81.5
1	115 hours	85
2	"	85
3	"	85

temperature investigation was stored at room temperature and observed simultaneously. It displayed no measurable loss in ESR signal intensity during the study.

Odor analysis of those samples exposed to 60°C and a control maintained at room temperature gave no qualitative or quantitative differences. The odor emitted by all the samples was the characteristic WDH odor.

However, for those samples exposed to 100°C for 115 hours the panel was in general agreement that, as opposed to a control, their odor was easily recognized as less intense.

b. ESR Signal Decay at 70, 80, and 90°C, Using Preparation 9

Samples of Preparation 9 were studied in closed quartz tubes which also served as sample holders for ESR observation, and the ESR signal was observed at various intervals for cumulative signal decay. Two samples, one in a nitrogen atmosphere and the other in an air atmosphere, were studied at each temperature. A description of the samples is given in Table 5.

Samples were placed in an oven controlled to ±2°C after initial ESR examination for unpaired spins. Samples were kept in the oven for an interval of 1/2 hour, then were removed, cooled, and re-examined to determine signal decay. The signal given by a coal standard was used to establish the stability of the spectrometer. The

TABLE 5. DESCRIPTION OF SAMPLES FOR FREE RADICAL DECAY STUDIES AT 70, 80, AND 90°C

Sample	Description
70A	0.090 g in tube. 0.046 g seen by spectrometer. Heated in air at 70°C.
70N	0.050 g in tube. All seen by spectrometer. Heated in nitrogen at 70°C.
80A	0.074 g in tube. 0.053 g seen by spectrometer. Heated in air at 80°C.
80N	0.049 g in tube. 0.038 g seen by spectrometer. Heated in nitrogen at 80°C.
90A	0.060 g in tube. 0.045 g seen by spectrometer. Heated in air at 90°C.
90N	0.067 g in tube. 0.047 g seen by spectrometer. Heated in nitrogen at 90°C.

cumulative decay of the signal with heating time is shown for the samples in Table 6.

Plots of the decay curves for all temperature studies (60, 70, 80, 90, and 100°C) are shown in Figure 14.

3. Odor Comparisons

An off-gas experiment was conducted as follows: A U-tube containing 0.123 grams of Preparation 9 in the bottom of the tube retained by glass wool was placed in an oven at 90°C for two hours. During this time, nitrogen dried in a dry ice-acetone trap was passed through the tube at the rate of 870 cc per minute. The out-gas was trapped at -196°C. After being returned to room temperature, the trap gave a fairly strong WDH odor and a very strong burnt odor. In addition, 10 mg of water were found condensed in the trap. An odor analysis on the residue was performed and is reported in the work immediately following.

Table 7 gives the results of an odor analysis by an informal panel of the heated samples 70A through 90N, an unheated control from Preparation 9, and of the residue from the off-gas experiment just described. The panel was asked to rate the intensity of the WDH odor, giving for the control an arbitrary value of 10.

4. Decay in Buffers of Varying pH

There are free radicals, such as peroxylamine disulfonate negative ion, that exhibit readily observable pH dependence in their

TABLE 6. PERCENT OF ESR SIGNAL REMAINING AFTER HEATING SAMPLES 70A TO 90N

Exposure Time	Sample					
	70A	70N	80A	80N	90A	90N
30 min	100.0	89.0	77.0	74.9	65.7	75.9
60 "	82.6	82.0	70.0	68.5		
75 "					34.4	66.3
90 "	80.6	78.7	66.7	64.5		
105 "					51.8	60.0
120 "	75.8	79.7	62.0	61.4		
135 "					50.5	60.1
2.5 hours	77.5					
4 "	73.6		53.6	52.8		
5.5 "					41.8	51.7
7.5 "			47.1	48.8		
8.5 "					37.1	43.0
12.5 "					32.2	37.0
68 "		42.9				
70 "	48.6					

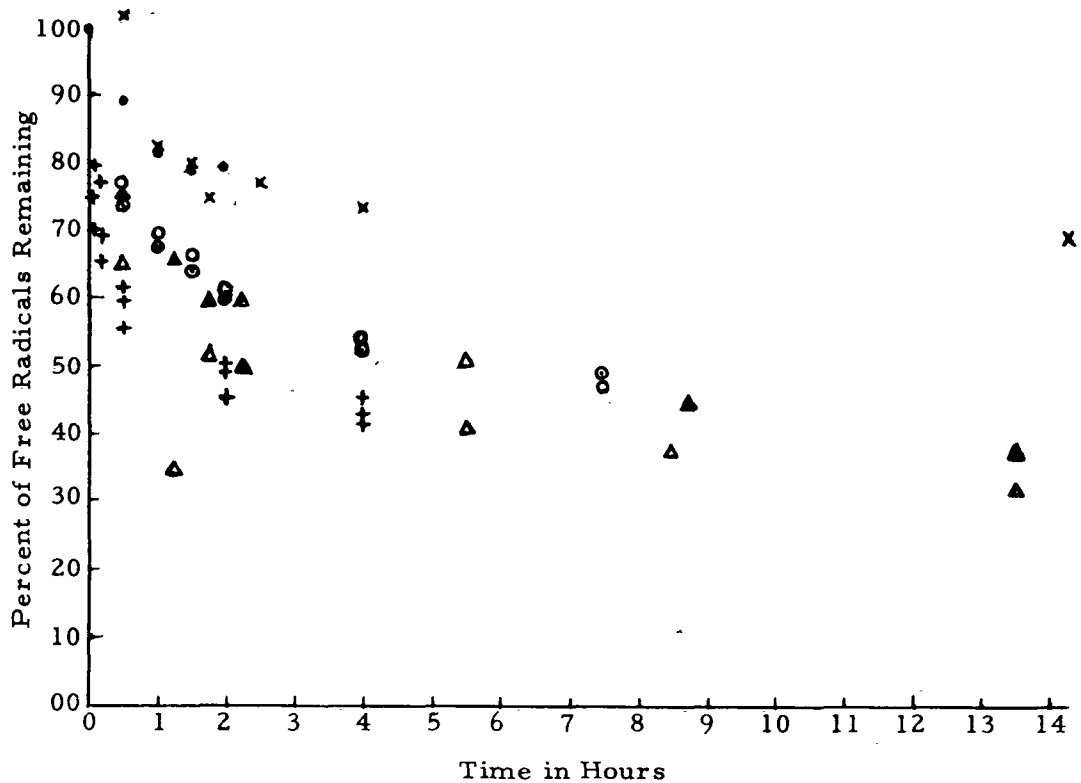


FIGURE 14. GRAPHICAL REPRESENTATION OF FREE RADICAL DECAY AT VARIOUS TEMPERATURES BOTH IN AIR AND IN NITROGEN

TABLE 7. ODOR ANALYSIS OF HEATED SAMPLES BY INFORMAL PANEL

Sample	Amount of Dry Material (mg)	Panel Members						Average Response
		B. S.	C. S.	D. M.	B. B.	G. M.	D. C.	
Preparation 9 unheated (Control)	10.0	10	10	10	10	10	10	10
Residue from off-gas experiment	11.0	12	8	15	10	15	7.5	11
70A	10.5	11	3	20	3	15	3	9
70N	10.7	11	7	5	5	5	1	6
80A	11.5	5	5	10	1	10	4	6
80N	9.5	7	5	10	1	15	3	7
90A	10.6	11	5	15	1	20	2	9
90N	11.0	8	7	5	1	15	8.5	7

stability in aqueous solution. An experiment was performed to determine if there was a pH at which the WDH odor could be released from a preparation made according to Hedin's procedure without effecting free radical decay. A series of buffer solutions was prepared. Only three of the solutions were used in the experiment as follows:

(1) 4.08 ml 0.10 M NaOH
5.0 ml 0.10 M H_3BO_3

pH measured with a Beckman Model GS pH meter was 9.42.

(2) 2.96 ml 0.10 M NaOH
5.00 ml 0.10 M KH_2PO_4

pH was 6.73.

(3) 4.85 ml 0.2 M HCl
2.50 ml 0.2 M KCl

pH was 0.83.

Samples of Preparation 9 prepared according to Hedin's procedure were weighed and dissolved in the buffer solutions as follows:

(1) 0.0312 g of Prep. 9 and 1 ml of solution (1) above. (pH = 8.75).

(2) 0.0204 g of Prep. 9 and 1 ml of solution (2) above. (pH = 6.70).

(3) 0.0282 g of Prep. 9 and 1 ml of solution (3) above. (pH = 3.08).

These samples were submitted to an informal panel for odor analysis. Note the buffering action of the irradiated material. Members of the panel were not in agreement on either the quantity or quality of odors

evolved. There was a tendency to assign the greatest intensity of odor to the sample at high pH, which would be exptected from the relative quantities of sample used. Two of the five panel members detected wet chicken feather or burnt chicken feather odor from the sample at high pH, and one panel member detected a burnt chicken feather odor instead of a WDH odor from the sample at low pH.

A series of samples were used for examination in the ESR spectrometer as follows:

(1) 0.0260 g Prep. 9
0.1401 g Solution (1) (pH 9.42)

(2) 0.0306 g Prep. 9
0.1198 g Solution (2) (pH 6.73)

(3) 0.0310 g Prep. 9
0.1504 g Solution (3) (pH 0.83)

Neither the samples nor buffer controls exhibited ESR signals. The pH of the solutions was not determined because of the small volumes. In view of the buffering action of the protein at lower concentration, however, still less of a spread in pH values would be expected than that determined for the solutions used in odor analysis.

C. Investigations Depending Primarily on Odor Analysis

1. Investigation of Material Made by Landmann's Procedure

Samples of chromatographic fractions prepared in accordance with the procedure set forth by Landmann's group at the American Meat Institute Foundation (Ref. 6) were irradiated in a Co^{60}

source to a dose of 4.6×10^6 rad. ESR signals were first obtained from the samples. Samples were then suspended in water and odor comparisons were made by the panel to ascertain the presence or absence of WDH odor and some estimate of relative intensity when present. Variation in both ESR signal and odor were observed. No WDH odor was noted in any of the samples. Table 8 presents the results of odor comparison.

A sample of the residue which remained after methanol extraction was also exposed to gamma irradiation to a dose of 4.6×10^6 rad. Odor evolved from this sample when water was added was identified as WDH by a majority of the panel. Several members of the panel did doubt that the odor was WDH and detected an acetic acid or sauerkraut component in the odor.

In the absence of WDH odor evolution by any of the fractions, there seems no point in presenting the low intensity ESR signals that were obtained from these fractions.

2. Vapor Phase Partition of WDH Odorants

The probable need for correlating the ESR signal decay data with the kinetics of odor production and for identification of the odorants prompted a brief investigation of volatiles from irradiated and unirradiated samples of Preparation 1 by vapor phase chromatography. A Perkin-Elmer Model 154D gas chromatograph with a six-foot column of Carbo-wax 1500 and Dow-Corning 200 on Chromosorb P was employed.

TABLE 8. ODOR COMPARISON OF CHROMATOGRAPHIC FRACTIONS OBTAINED BY LANDMANN'S PROCEDURE

Fraction	Sample Weight (gm)	Odor
4, 5, 6, 7 (between yellow bands)	0.013	Soap, rancid fat
8, 9 (part of second yellow band)	0.052	Fish
10 (part of second yellow band)	0.041	Fish
11	0.031	Similar to 4, 5, 6, 7
12, 13	0.029	"
14, 15	0.014	"
16	0.006	"
17, 18	0.013	"
19, 20	0.009	"
21, 22	0.007	"
23, 24	0.009	Similar to 4, 5, 6, 7, with trace of fish
25, 26, 27	0.012	Different from others, but no WDH odor

The column temperature was 40°C and thermal conductivity detection was employed. Five grams of each sample were dissolved in 80 ml of water and the volatiles were stripped from these solutions at a pressure of 40 ml of mercury and a temperature of 38°C. The volatiles were collected on a preliminary column about eight inches in length of similar composition to that in the gas chromatograph. The preliminary column was heated in a bath to 40°C in order to drive off the volatiles. Chromatograms of the irradiated and unirradiated samples from Preparation 1 showed differences that were not interpreted. With respect to the irradiated sample, it was possible to sample the output of the machine by smell, and no decided odors that might be considered characteristic of the WDH odor or contributing to the WDH odor could be detected with certainty. Immediately following the detection of water vapor, WDH odor was detected at the output from the preliminary column. Since the WDH odorants have a greater retention time than water, the vapor phase chromatography equipment on hand was inadequate to deal with them. The development of the necessary columns would have required an additional program.

There was similar experience with Preparations 3, 4, and 5. For Preparation 5, a one-gram sample was employed. After transferring volatiles preceding water from the preliminary column to the main chromatographic column, the preliminary column was heated from -196°C to 50°C, opened to the atmosphere, and allowed to elute.

After 10 minutes had elapsed, WDH odor was detected and this odor was introduced into a trap at -196°C. Mass spectroscopic examination of the trapped contents revealed only water. A second odor similar to WDH was observed after a second 10-minute lapse, but mass spectroscopy once again could reveal nothing but water.

Because the separation of the odorants appeared to be so marked, repeating the procedure several times with separate apparatus for confirmation seemed desirable. To achieve this, an 8-inch column was prepared utilizing a coating of β,β'-oxypropionitrile (obtained from American Cyanamid) on 60 - 80 mesh Gas Chrom "P" (supplied by Applied Science Lab., Inc.). The β,β'-oxypropionitrile comprised 15% of the total weight of the coated material. The Gas Chrom "P" was purchased acid washed, followed by alcoholic base wash. Methanol was the solvent employed in coating. Figure 15 depicts the laboratory apparatus. Dry nitrogen gas was used as the carrier, and the reaction vessel was such that one could add water and strip odorants while excluding oxygen entirely, in addition to minimizing odorant losses. Two three-way stopcocks, (A) and (B), were provided to by-pass the stripping vessel following the removal of volatiles along with three-way stopcock (C), which enabled one to exercise on-stream odor monitoring. The water bath temperature was thermostatically controlled at 50°C, and the column was immersed in liquid nitrogen. A condenser was included in the system to minimize the amount of water carried over

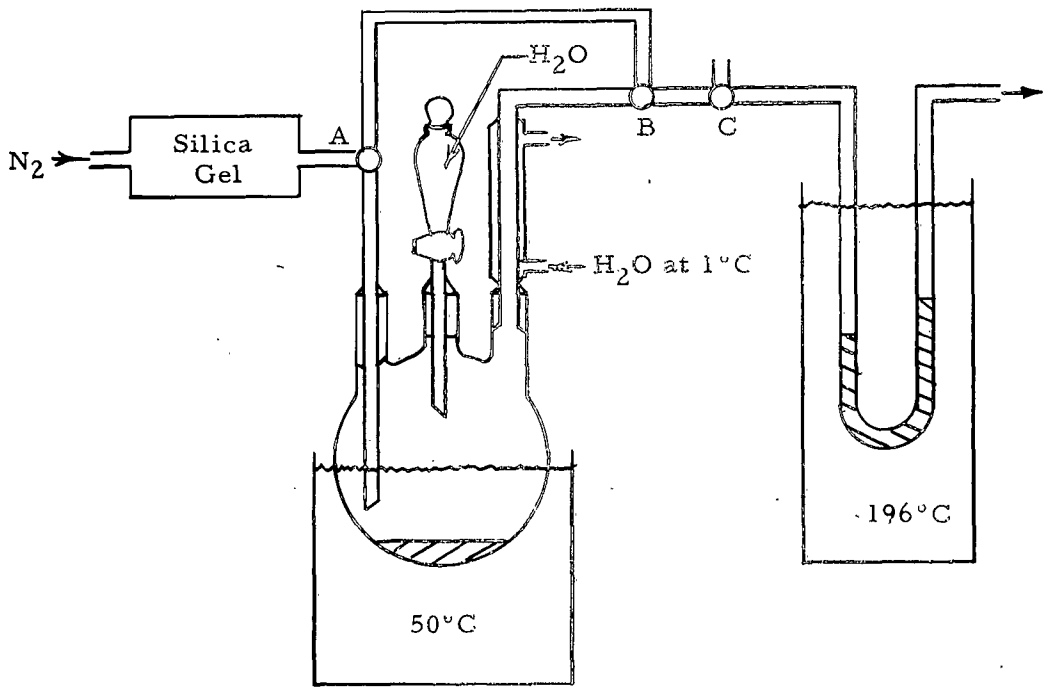


FIGURE 15. APPARATUS FOR VAPOR PHASE PARTITION OF VOLATILES FROM IRRADIATED BEEF FRACTION

to the column, and the flow rate of the carrier gas was adjusted to 40 cc/min.

The attempt to fractionate the odorants from Preparation 5 was performed in an all-glass system with the exception of a one-inch piece of Tygon tubing which was utilized to butt the glass tubing to the gas regulator. It was our hope to establish an accurate time relationship with the appearance of fractionated odorants. One and one-half grams of the sample, gamma irradiated to 4.6×10^6 rad, were placed in the stripping flask, followed by the purging of the system for 15 minutes with nitrogen gas. The sample was then moistened with 10 ml of water, and within 30 seconds a very strong odor resembling burnt fat (no obvious WDH odor) was detected at stopcock (C). It was immediately closed to the atmosphere and opened to the column. The gas flow, maintained at 40 cc/min, was briefly monitored at stopcock (C) every 15 minutes. After 2-1/2 hours, strong odor was still being stripped from the sample. At this point, stripping was discontinued, and stopcocks (A) and (B) were positioned to by-pass the stripping vessel while stopcock (C) remained closed to the atmosphere. At time zero, the column temperature was elevated from -196°C to 50°C. It was found that after approximately 30 seconds a very strong odor similar to burnt fat was evolved. This odor continued until three minutes after time zero. During the next two minutes, a faint sweet odor was observed, after which no odor was detected until nine minutes after

time zero. From 9 through 12 minutes following time zero, a faint WDH odor was recognized. Following this, the column exhaust continued to emit odorants similar to burnt fat, until the gradual disappearance of odor at about 55 minutes after time zero.

3. Odor Comparisons of Methanol Extracts from Preparations 8 and 9

Several of the first methanol extracts made from irradiated samples of Preparations 8 and 9 gave the caramel odor but evolved no WDH odor with the addition of water. These extracts were dried under vacuum produced by an aspirator, and the consequent exposure to water vapor during drying is believed to explain this discrepancy. Indeed, during one such drying, WDH odor was detected at the output from the aspirator.

A Soxhlet extraction of 1.61 grams of irradiated Preparation 8 was carried out for approximately 8 hours with methanol dried over calcium sulfate. The extract was dried with a stream of nitrogen. The methanol removed was passed through a dry ice-acetone trap. The methanol removed had a slight caramel odor. The dry residue from the extraction gave no WDH odor upon suspension in water. The dry extract weighed 0.053 grams and had a caramel odor. WDH odor was evolved from a heated suspension of the extract in water made alkaline by the addition of calcium oxide.

An irradiated sample of Preparation 9 was extracted with hexane in a Soxhlet extractor. The dry residue was extracted with methanol at room temperature. The sample, weighing 1.557 grams, yielded 0.007 gram of methanol extract, or 0.4%. This was a brown, waxy material with a caramel odor. On the addition of water, a WDH odor like that from the original preparation was evolved. The dried residue remaining after the methanol extraction gave no WDH odor after treatment with water, but gave an odor similar to the odor of beef broth made from the dehydrated preparations used for preparing meat broths in bacteriology.

The odor of the residue from another methanol extract of an irradiated sample of Preparation 9, when treated with water, gave a very irritating odor similar to that of hydrogen peroxide or oxides of nitrogen. After standing a few minutes, the odor was that of meat broth. The extract from this sample also gave the WDH odor.

The odors noted were laboratory observations and no test panel confirmation was made.

A small sample of a methanol extract from 1.8 gm of unirradiated Preparation 9 was prepared. This material was similar in appearance to the irradiated extract, but had a beef-broth odor rather than the caramel odor. A larger sample would be required for chromatographic separation.

IV. CONCLUSIONS AND RECOMMENDATIONS

The variable and preliminary nature of the data do not support conclusions upon which to base a firm mechanism for the production of WDH odor. None of the findings casts doubt that a free radical mechanism is involved in WDH odor production, but there is no direct proof that there is an essential free radical step in the production by ionizing radiation of WDH odor. Inability to repeat apparently significant findings that occurred early in the program proved especially expensive of effort. First, there was the observation that ESR signals from Preparations 1 and 4 from high fat beef, and from Preparations 2 and 3 from low fat beef, displayed respective similarities; but any hope for a meaningful correlation between fat content of the preparation and ESR signal shape was destroyed by the resemblance of the signal from high fat Preparation 5 to those from low fat Preparations 2 and 3. Second, during dialysis of low fat Preparation 2, a grayish-white precipitate appeared in the dialysis tube which dried under vacuum to a yellow coating resembling shellac and weighing 593 mg. Approximately half of this was irradiated in air. This and the other half, which was maintained as an unirradiated control, were sent sealed in evacuated tubes to Hedin for odor analysis. The irradiated sample gave a strong WDH odor upon the

addition of water, with a transient boüillon odor, while the unirradiated control gave a bouillon odor with a burnt overtone. The material seemed to him of relatively low molecular weight (Ref. 15). Earlier samples from the non-dialyzable fraction of Preparation 2, which had not precipitated but had been lyophilized as usual, had been sent to Hedin for odor analysis along with samples from Preparations 3 and 4. All of the irradiated samples gave WDH odor, and none of the unirradiated controls did. Significantly, perhaps, the irradiated sample from Preparation 2 gave a relatively weak WDH odor with some wet chicken feather odor, whereas the other two gave stronger odors characterized merely as WDH (Ref. 16). Low-fat Preparation 3 gave a similar precipitate, but the sample was lost. Although further preparations did not demonstrate quite the same behavior with respect to precipitation, there appears a strong possibility that a fractionation of value can be performed in this manner.

There is yet another failure to reproduce a result, which at present seems to bear more directly upon the problem at hand than the two just mentioned. This is the failure to obtain a fraction yielding WDH odor through following Landmann's procedure (Ref. 6). In general, the group at the American Meat Institute Foundation treated pre-irradiated beef and obtained a chromatographic fraction (Fraction 5) which gave rise to WDH odor with the addition of water. Had unirradiated beef not been fractionated, and had the same Fraction 5 not given rise to WDH odor upon irradiation, then the identity of this fraction with a

precursor of WDH odor would have been of doubtful validity. Fraction 5 did develop WDH odor, however, and the fractionation presumably has meaning in terms of isolating a precursor. The observation was made, however, that an additional fraction (Fraction 7) resulting from a straight methanol wash of the column also gave a "quite intense" WDH odor after standing overnight in methanol suspension before dilution of the fraction in water (Ref. 6). A similar failure to extract WDH-odor-producing fractions by methanol extraction from his own unirradiated material was experienced by Hedin (Ref. 17). He used both methanol and n-butanol in Soxhlet extraction of both the crude aqueous extract and the 26 - 50% ammonium sulfate precipitable fraction of it. Irradiation of the dry residues gave WDH odor as it had before extraction, whereas irradiation of the dry extracts gave no WDH odor.

At this point a statement of faith must be made: WDH odor, at least from Hedin's material, is believed to arise from protein, and a specific grouping of amino acids seems to invite the greatest suspicion. Consider certain of Hedin's observations:

(1) Collagen is a model source of WDH odor (Ref. 1). Collagen contains no cysteine or cystine, but contains 0.8% methionine, 27.2% glycine, and 14.1% hydroxyproline (Ref. 18).

(2) Methionine plus ninhydrin plus heat at pH 5 gives an odor that resembles true irradiation odor (Ref. 19). Surely, therefore, methional is an important component of WDH odor. The work of the

MIT group gives this same impression (Ref. 5). Methionine seems the obvious source of methional from Hedin's material and from beef generally.

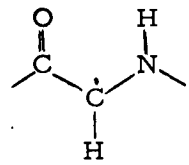
(3) No correlation could be found between the loss through irradiation of any one amino acid and the appearance of WDH odor (Ref. 2). This observation does not exclude correlation between the loss of a group of closely associated amino acids and the appearance of WDH odor. There seems no reason that the group could not be as small as a dipeptide.

(4) Of three fractions of Hedin's crude material giving (a) WDH odor, (b) slight WDH odor, and (c) no WDH odor, the amino acids showing diminishing concentrations with diminishing odor productivity were arginine, hydroxyproline, proline, glycine, and alanine (Ref. 2). (The hydroxyproline only was entirely absent from the fraction giving no WDH odor. This interesting observation can only be ignored for the present, since no other data has been found that would relate it to this investigation.)

Although the existence of a free radical mechanism in the production of WDH odor by ionizing radiation has not been proven, it should be considered probable. Every case of free radical decay has proven a case of the production of WDH odor, and vice versa. Also, in every case of the appearance of WDH odor, the presence of water has been either certain or probable. As a working hypothesis, therefore,

WDH odor is considered to arise from the reaction of water at free radical sites in protein, the free radicals being long-lived in dry protein and only transient in whole beef. In whole beef, of course, indirect action may alter the reaction mechanism in that the primary action of radiation need not be the induction of a free radical in the protein itself. The most probable site for long-lived free radicals in the dry material that might react with water to give WDH odorants is considered to involve both glycine and methionine. The ESR evidence points directly to involvement of glycine, although this evidence was clear only in the cases of Preparations 1 and 4. There is no direct ESR evidence for the involvement of methionine, but an oxygen-stable free radical is known to arise from the irradiation of glycyl-DL-methionine (Ref. 11). Whether the radiation-induced ESR signal from this substance would fit any of those obtained from Hedin's material is unknown, but the possibility seems worth investigating.

Therefore, with no direct evidence to support it, the suggestion is advanced that WDH odor partially arises from sites in protein at which glycine and methionine are either bonded, or at least are near neighbors. It would be foolhardy to suggest that the components of WDH odor arise only from these sites. At these sites, the primary action of radiation would probably be to remove one a proton from glycine, giving a free radical



The fate of the removed proton and the subsequent action of added water to give methional and other products could be decided if the suggested reaction site should prove correct. Proof of correctness could be time consuming, for an obvious direct approach would involve fractionation by partial hydrolysis and chromatography of Hedin's crude material, with odor analysis and amino acid analysis of all fractions until finally the minimum peptide giving WDH odor could be completely described. (In this connection, characterization of fractions that do not give WDH odor is quite as important as characterization of those that do. Amino acid analyses reported by Hedin (Ref. 2) of several fractions that both did and did not give WDH odor seemed most instructive.) Such work was begun by Hedin employing fractional electrical transport on the dialyzable parts of tryptic and 8M urea digests of the 25 - 50% ammonium sulfate precipitable fraction of his crude material (Ref. 1), with encouraging results. An initial test of the suggestion might involve the irradiation and odor analysis of several other proteins that contain either methionine or glycine, but not both. Edestin contains methionine but no glycine; fibroin (silk), salmin (protamine), calf liver histone, and ox insulin contain glycine but no methionine. None of these proteins should give

WDH odor upon irradiation. Edestin, except for its apparent lack of hydroxyproline and perhaps of hydroxylysine provides a crucial test (Ref. 18). Perhaps other proteins of known amino acid sequence could be found which contain glycine and methionine, both bonded and separated. Thus β-corticotropin from hog ACTH, which contains one methionine unit and three glycine units in a total of 39 units in its chain, would not be expected to yield WDH odor because five units separate the methionine unit from the nearest glycine unit. In applying these tests, irradiation in the dry state would be preferable because irradiation in solution or suspension might lead to different results. In aqueous suspension, for example, any methionine-containing protein might be found capable of yielding WDH odor, even though this might not be true for irradiation in the dry state.

There are two other findings that seem worthy of some consideration. First, WDH odorants appear extractable from Hedin's crude material by methanol following irradiation; but if the methanol extraction is performed prior to irradiation, the irradiated extract gives no WDH odor, whereas the irradiated residue does. This indicates that the WDH odor precursor in Hedin's material is not methanol extractable, whereas the WDH odorants themselves are methanol extractable. Second, the WDH odorants apparently can be removed along with other substances from Hedin's crude irradiated material at 90°C in a nitrogen stream with subsequent trapping at liquid nitrogen temperature.

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